Novel inhibitors of steroidogenesis for the treatment of hormone-dependent breast cancer

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NOVEL INHIBITORS OF STEROIDOGENESIS FOR THE TREATMENT OF HORMONE-DEPENDENT BREAST CANCER

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Abstract

Inhibition of steroidogenic enzymes, which are involved in the biosynthesis of active estrogens, represents an attractive approach to treating hormone-dependent breast cancer. Synthetic routes to novel steroid-based inhibitors of two key enzymes, steroid sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1), are described. Biological evaluation of the derivatives *in vitro* indicated that most of the compounds synthesised were active against the selected targets to varying degrees. Particular attention was given to assessing the estrogenicity of potential inhibitors, since agonist activity at the estrogen receptor is undesired in the treatment of estrogen-dependent pathologies.

Modifications to the D-ring of estrone-3-*O*-sulfamate EMATE, a potent STS inhibitor, successfully afforded a 3-sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide template, from which a series of *N*-alkylated analogues were prepared. The *N*-propyl and *N*-pyridin-3-ylmethyl derivatives were 18 times more potent than EMATE *in vitro*, with IC_{50}s of 1 nM in placental microsomes. *In vivo*, these compounds inhibited 99% of rat liver sulfatase activity at an oral dose of 10 mg/kg. Importantly, they were also devoid of estrogenic activity. The SAR for the derivatives synthesised is discussed and a QSAR was established.

Substrate-based design of 17β-HSD type 1 inhibitors resulted in the identification of several lead compounds, mostly D-ring modified estrogens. The potential of 16-alkylidenes derivatives of estradiol as enzyme-generated irreversible inhibitors was examined. Small modifications to the estra-1,3,5(10)-triene nucleus were investigated. Both studies were assisted by molecular modelling, using the crystal structure of the enzyme. D-Ring fused heterocyclic derivatives of estrone were prepared and an SAR was built around *N*-alkylated 16,17-fused pyrazoles. The *N*-ethoxymethyl derivative emerged as a potent inhibitor of 17β-HSD type 1 *in vitro* without being estrogenic. A preliminary study focused on the design of dual inhibitors of STS and 17β-HSD type 1 is also reported here. The synthetic work was supported by single crystal X-ray analyses.

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Publications

Some of the work described in this thesis has appeared in the following publications:

Fischer, D. S.; Woo, L. W. L.; Mahon, M. F.; Purohit, A.; Reed, M. J. and Potter, B. V. L. D-Ring modified estrone derivatives as novel potent inhibitors of steroid sulfatase. *Bioorg. Med. Chem.* **2003**, *11*, 1685-1700

Fischer, D. S.; Chander, S. K.; Woo, L. W. L.; Fenton, J. C.; Purohit, A.; Reed, M. J. and Potter, B. V. L. Novel D-ring modified steroid derivatives as potent, non estrogenic, steroid sulfatase inhibitors with *in vivo* activity. *J. Steroid Biochem. Mol. Biol.* **2003**, *84*, 343-349

Abbreviations

Å	Angstrom (10^{-8} cm)
Ac	acetyl
app	apparent (spectral)
aq.	aqueous
Ar	aryl
Bn	benzyl
br	broad (spectral)
Bu	butyl
^{t}Bu	*tert*-butyl
°C	degrees Celsius
ca.	approximately
CA	carbonic anhydrase
cm	centimetre(s)
COUMATE	4-methylcoumarin-7-*O*-sulfamate
δ	chemical shift
d	doublet (spectral)
DBD	DNA binding domain
DCM	dichloromethane
DEPT	distortionless enhancement by polarisation transfer
DMA	dimethylacetamide
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
E1	estrone
E2	estradiol
E3	estriol
e.g.	for example
EMATE	estrone-3-*O*-sulfamate
eq.	equivalent
ER	estrogen receptor
Et	ethyl
f	femto

FAB	fast atom bombardment (mass spectrometry)
G	gram(s)
h	hour(s)
HBD	hormone binding domain
HDBC	hormone-dependent breast cancer
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectroscopy
HRT	hormone-replacement therapy
17β-HSD	17β-hydroxysteroid dehydrogenase
Hz	Hertz
IC_{50}	concentration causing 50% inhibition
IPA	isopropyl alcohol
IR	infrared
J	coupling constant (spectral)
Kg	kilogram(s)
Ki	inhibition constant
Km	Michaelis-Menten constant
L	litre(s)
LBD	ligand binding domain
lit.	literature
μ	micro
m	milli, multiplet (spectral)
M	moles per litre, molecular ion (mass spectrometry)
m/z	mass to charge ratio
Me	methyl
MHz	megaHertz
min	minute(s)
mol	mole(s)
mp	melting point
MS	mass spectrometry
ν	spectral number (infrared spectroscopy)
n	nano

NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
nd	not determined
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
pH	logarithm of the concentration of H^+
pK_a	logarithm of the acidity constant
p.o.	per oral
ppm	parts per million
q	quartet (spectral)
QSAR	quantitative structure-activity relationship
rbc(s)	red blood cell(s)
rt	room temperature
R*f*	retention factor
Rt	retention time
s	singlet
SAR	structure-activity relationship
s.c.	subcutaneous
S.D.	standard deviation
STS	steroid sulfatase
t	triplet (spectral)
TBAF	tetrabutylammonium fluoride
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	tetramethylsilane
vs.	versus
wt.	weight

Trivial/Approved names of steroid

Androstenedione	4-Androstene-3,7-dione
Cholesterol	5-Cholesten-3β-ol
Dehydroepiandrosterone	5-Androstene-3β-ol-17-one
Estradiol	Estra-1,3,5(10)-triene-3,17β-ol

Estriol	Estra-1,3,5(10)-triene-3,16α,17β-triol
Estrone	3-Hydroxy-estra-1,3,5(10)-triene-17-one
Progesterone	4-Pregnene-3,20-dione
Pregnenolone	3β-Hydroxy-5-pregnene-20-one
Testosterone	17β-Hydroxy-4-androstene-3-one

Contents

To my parents

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Introduction

Breast cancer is a disease of major importance which still remains a leading cause of death among women in most Western countries. It is estimated to affect approximately 1 million women per year across the globe.[1]

Britain has one of the highest incidence of breast cancer in the world with over 40,000 patients diagnosed each year, accounting for nearly 1 in 5 of all cancer cases. It is estimated that 1 in 10 women living to the age of 85 in Britain will develop breast cancer during the course of their lives.[2] Although modern methods of treatment as well as an earlier detection of the disease have greatly improved survival rates, breast cancer remains the commonest cause of death for women aged between 40-50.[3]

All women are randomly at risk of breast cancer, although a number of risk factors have been identified, most of them related to a woman's hormonal and reproductive history as well as their family background of the disease. Hormones, in particular estrogens, play a major role in the promotion and development of breast cancer and currently between one and two-thirds of all breast carcinoma rely on estrogens for their sustained growth. This particular type of breast cancer, known as Hormone-Dependent Breast Cancer (HDBC), predominantly affects postmenopausal women who account for nearly 80% of all cases.

The past decade has witnessed the accelerated development of new drugs and strategies to fight HDBC, and several compounds have entered clinical trials or are already marketed. They are all designed to deprive the tumours of estrogens by either competing with the natural hormones for specific binding sites, or by blocking their biosynthetic pathways. However, there are still significant limitations to existing treatments and cancer therapy is often rigorous, debilitating and uncomfortable. Thus, when designing new drugs, not only the quantitative aspects, in terms of increased survival, but also the qualitative value, in terms of the well-being of the patient, must be considered.

As a result, drugs that are more selective, less invasive and ideally devoid of side-effects need to be designed to efficiently combat breast cancer. In order to achieve

this goal, it is necessary to understand the underlying mechanisms of hormone action, in particular that of estrogens, as well as their implication in carcinogenesis.

1.1 Hormones: the body's chemical messengers

Intercellular communication is of paramount importance in the maintenance of life for all complex organisms. In vertebrates, including humans, there are two major networks of internal communication which are intimately connected: the endocrine system and the nervous system. Together, they provide for full coordination of physiological responses and regulation of the body's chemistry.

In order to communicate, cells use chemical messengers, i.e. extracellular signalling molecules, which bind to specific receptors of a target cell. The biological response to a messenger is therefore mediated through receptors, which are membrane-bound, cytoplasmic or nuclear proteins. In the nervous system, neurotransmitters released under electric impulse provide a fast and direct communication with neighbouring nerve cells. In the endocrine system, hormones are delivered by the circulatory system to act at distant sites, offering a slower and more widespread mode of communication. The complementary function and synergistic action of the two systems is the key to a comprehensive control and adaptation between the body and its internal and external environment.[4]

1.1.1 Historical perspective

At the end of the 19th century, the concept that some organs were able to liberate into the bloodstream substances which assist in regulating body functions slowly emerged, challenging the existing view that coordination was the sole property of the nervous system.

In 1855, the French physiologist Claude Bernard first introduced the term 'internal secretion' when describing the glycogenic function of the liver and its ability to regulate blood sugar levels.[5] Forty years later, the first hormone was isolated by two

English physiologists, George Oliver and Edward Schaefer whose work on extracts of the adrenal gland led to the discovery of adrenaline.[6] Meanwhile, the existence of a second hormone was being revealed as Bayliss and Starling discovered secretin, a hormone capable of stimulating the secretion of pancreatic substances.[7] In 1905, during a lecture entitled 'The Chemical Correlation of the Functions of the Body', Starling introduced for the first time the term hormone.[8] The word derives from the Greek verb 'hormao' and means 'to excite'.

Since then, a large number of hormones have been isolated, characterised and synthesised. Some discoveries have had a dramatic impact worldwide, as exemplified by the isolation of insulin by Frederick Banting and Charles Best in 1921, which revolutionised the treatment of diabetes and the prevention of its complications.[9] A century after the discovery of the first hormone, thanks to the efforts of scientists in the field of endocrinology, we now benefit from a comprehensive understanding of the nature, mechanism of action and regulation of hormones and how the endocrine system functions in integrating the activity of diverse cells and organs.

1.1.2 General principles

Hormones play an essential role in a wide range of physiological and pathological processes. In general, they coordinate body functions to maintain a constant internal environment (homeostasis) but certain hormones also affect behaviour, and thus influence response to external environmental conditions. Five principal areas of hormonal function have been defined. They include the maintenance of physiological conditions, the general growth and development of the body, the control of reproduction, the different behaviour patterns and the control of energy metabolism (production, utilisation and storage).[4]

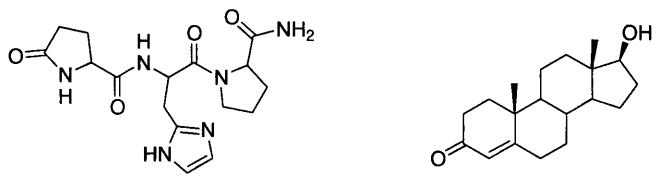
Hormones are produced by ductless internal glands or groups of secretory cells and are then discharged directly into the bloodstream in response to a stimulus. When hormones are released into the blood stream, they are carried via the circulation to other parts of the body where they act on remote target cells. The response to a

hormone is dependent on two factors: different hormones activate different cellular mechanisms, and not all cells have receptors for all hormones. As a result, some hormones limit their effects to specific body tissues, others act in a more general manner, stimulating a response throughout the body. In any case, the hormone interaction with the cell depends on the chemical type of hormone.

1.1.3 Chemistry of hormones and mechanism of action

The two major classes of hormones in the body, peptides and steroids, differ in their chemical structures and mode of action at their target cells.

The peptide hormones range in size from a tripeptide (e.g. thyrotropin releasing hormone or TRH, Figure 1.1) to a polypeptide containing nearly 200 amino acids (e.g. growth hormone). Many peptide hormones are synthesised from larger precursor proteins called prohormones. Theses are specifically cleaved by enzymes to give rise to functional hormones. Peptide hormones, because of their polarity, cannot pass through cell membranes and rely on an intracellular second messenger to elicit their response. They bind with high affinity to protein receptors located on the surface of target cells which in turn causes a conformational change in the receptor. This activates the production of a second messenger, which eventually leads to protein phosphorylation and results in the physiological response to the hormone.[10]



TRH Testosterone

Figure 1.1 Structures of a peptide hormone (TRH) and a steroid hormone (Testosterone).

The steroid hormones are generally smaller than peptide hormones and can be grouped into five families: the glucocorticoids, the mineralocorticoids, the estrogens, the androgens (e.g. Testosterone, Figure 1.1) and the progestins. Steroid hormones do not bind to cell surface receptors; since they are lipohilic, they easily pass through

the plasma membrane of their target cells and complex with specific receptors in the cytoplasm or the nucleus. In the nucleus, the hormone-receptor complex binds specific regions of the DNA and affects gene expression. The action of the proteins resulting from the transcribed genes produces the response to the hormone.[10]

The physiological response to a hormone is also related to its chemical type. The action of peptide hormones is quite rapid – from seconds to minutes, as compared to the slower action of steroid hormones. In general, peptide hormones allow the organism to make swift, adaptive response to a changing environment while steroid hormones have a longer-lasting maintenance function.

1.1.4 The endocrine system: control and regulation of hormone release

The endocrine system is composed of specialised cells, organised into glands and cell clusters which secrete hormones into the circulation. In order to maintain the correct regulatory function of a hormone, the endocrine system receives constant feedback information about the state of the system being regulated.

The secretory activity of most endocrine glands is controlled by the anterior pituitary (or adenohypophysis), which is the dominant gland of the endocrine system (Figure 1.2). It is located at the base of the hypothalamus, which functions as an important link between the nervous and the endocrine system. The hypothalamus receives messages from the brain via nerve terminals eliciting the release of hormones into the pituitary blood supply (hypothalamic releasing hormones), which in turn stimulates the release of pituitary hormones (trophic hormones).[4,11] The trophic hormones act as messengers to other target glands, providing instructions for the release of specific hormones (peripheral hormones). The hypothalamus and pituitary gland therefore work jointly, as a functional unit, to coordinate the endocrine and nervous systems in their actions.

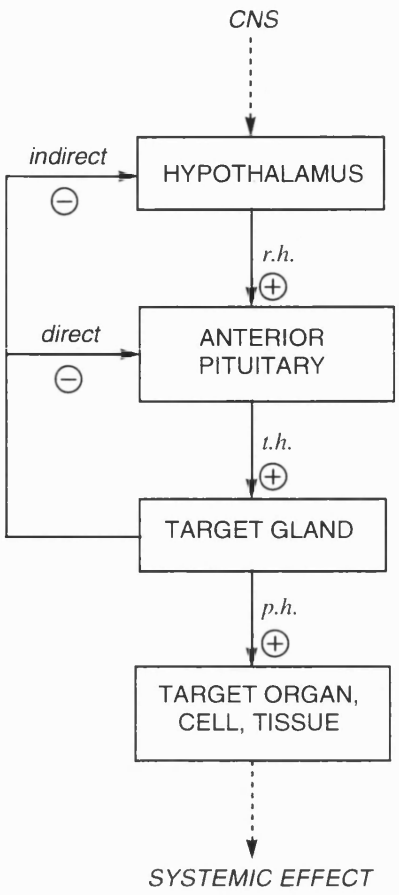


Figure 1.2 Schematic representation of inter-relations between the hypothalamic-pituitary hormones and peripheral hormones: direct and indirect negative feedback (r.h., releasing hormone, t.h., trophic hormone, p.h., peripheral hormone; CNS, central nervous system; + and – indicate stimulating and inhibiting effects respectively).

The major hormone-producing regions of the human body, besides the hypothalamic-pituitary axis, are the thyroid gland, the parathyroids, the pancreas, the adrenal glands and the gonads. The production and output of hormones of most of these endocrine organs is under the regulatory effect of the anterior pituitary and is regulated via a very sensitive feedback system (Figure 1.2). Regulation of hormone release occurs most commonly by negative feedback, where an increase in the level of a circulating hormone decreases the secretory activity of the cells producing it.

There are two types of negative feedback: the *direct* negative feedback is the most common 'closed-loop' control mechanism[4] which occurs when the levels of the target gland hormone exerts an inhibitory effect on the release of pituitary hormones.

The target gland hormone can also inhibit the release of pituitary hormone via *indirect* feedback, i.e. by inhibiting the secretion of hypothalamic releasing hormone. Positive feedback is less common, but a hormone may stimulate pituitary or hypothalamic hormone release to facilitate its own secretion. There are many examples of endocrine feedback loops throughout the body and for instance, the secretion of thyroxine by the thyroid gland is directly controlled by the trophic hormone TSH (thyroid stimulating hormone). Similarly, the corticosteroid hormones, secreted by the adrenal glands, indirectly inhibit the release of ACTH (adrenocorticotrophic hormone) from the pituitary, by modulating the secretion of hypothalamic CRH (corticotrophin releasing hormone).

1.2 Estrogens, the female sex hormones

Estrogens belong to the steroid hormones, a class of chemical messengers which mediate a wide variety of vital physiological functions, ranging from the anti-inflammatory response to the regulation of events during pregnancy.

Estrogens are responsible for all female secondary sex characteristics. They are concerned with the normal growth and development of the female reproductive system, breast development as well as the typical female body shape. In addition, they are involved in calcium and nitrogen metabolism,[12] and they are of critical importance in bone development, the maintenance of bone density and have cardioprotective effects.[13] There is also evidence to suggest that estrogens might have neuroprotective effects and are linked to cognitive functions.[14]

The first estrogen was isolated in 1929 by Edward Doisy and coworkers[15] who successfully crystallised estrone from urine extracts of pregnant women. This was followed by the discovery of estradiol in 1936. Although a number of important sterols including cholesterol, cholic acid and ergosterol had already been isolated in the 19th century, these two discoveries had a major impact on the field of endocrinology by opening it up to research in organic chemistry.

1.2.1 Biosynthesis of estrogens

All steroid hormones are biologically derived from cholesterol and have in common a cyclopentanoperhydrophenanthrene ring structure (Figure 1.3). Most of these hormones are synthesised and secreted into the bloodstream by the adrenal cortex and the gonads (ovaries and testes). The series of reactions involved in the biosynthesis of estrogens is summarised in Figure 1.4.

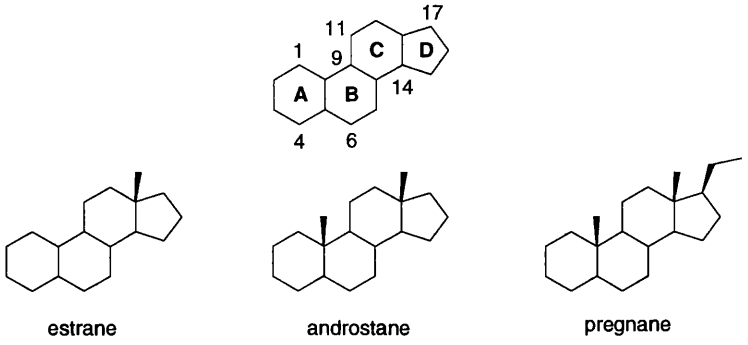


Figure 1.3 Numbering of the steroidal skeleton, designation of the four rings and names of steroid nuclei possessing 18, 19 and 21 carbons.

i) From cholesterol to steroids

Besides being ingested in food, cholesterol is synthesized in the body from acetylcoenzyme A, a biological source of acetate, via a multi-step pathway. Cholesterol is stored inside fat droplets in the cell cytoplasm and its breakdown into steroids is regulated by hormonal signals.[12] Most of the enzymes involved in the steroidogenesis are cytochromes of the P_{450} family.

Pregnenolone is the first steroid to be formed from cholesterol and its biosynthesis corresponds to the rate-controlling step in the steroid cascade.[12] This 21-carbon steroid is obtained after excision of a six carbon unit from cholesterol by the cytochrome P_{450} side chain cleavage enzyme. To yield the steroids, a series of reactions occurs at specific sites: pregnenolone is converted into glucocorticoids and mineralocorticoid in the adrenal cortex, while conversion into androgens and estrogens takes place in the testes and the ovaries respectively.[16]

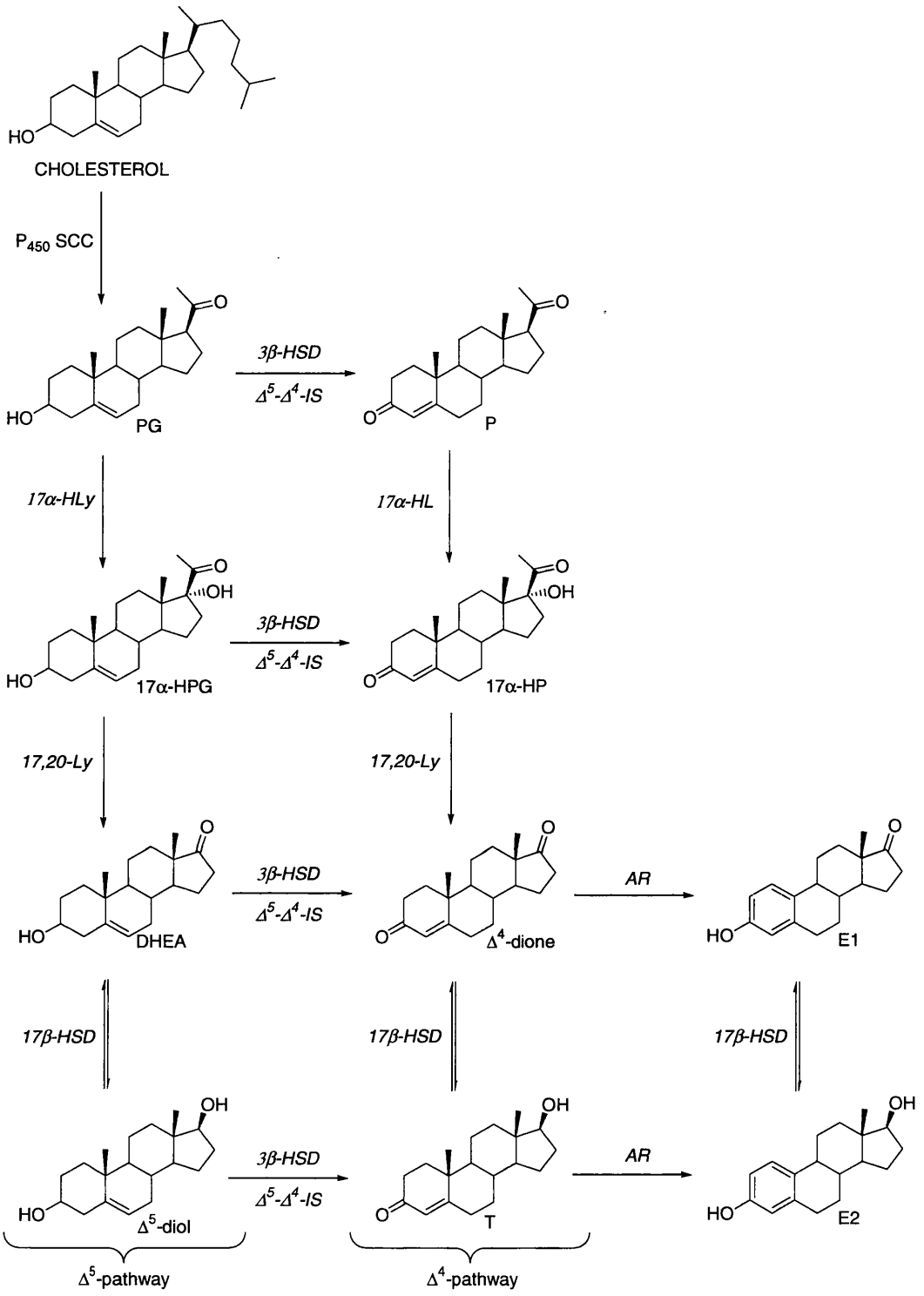


Figure 1.4 Summary of the pathways leading to estrogens. The abbreviation for the enzymes are: P_{450} SCC, P_{450} side-chain cleavage; 17α-HLy, 17α-hydroxylyase; 3β-HSD/Δ^5-Δ^4-IS, 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5-isomerases; AR, aromatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase. The abbreviations for the steroids are: PG, pregnenolone; P, progesterone; 17α-HPG, 17α-hydroxy-pregnenolone; 17α-HP, 17α-hydroxyprogesterone; DHEA, dehydroepiandrosterone; Δ^4-dione, 4-androstene-3,17-dione; Δ^5-diol, 5-androstene-3β,17β-diol; T, testosterone; E1, estrone; E2, estradiol.

ii) Pathways to estrogens

The biosynthesis of estrogens mainly occurs in the ovaries during the reproductive years of a woman's life. The pathway from pregnenolone to estrogens can be divided into two basic stages, each taking place in specialised cells of the ovarian follicle: the production of androgens in the theca cells and the conversion of androgens into estrogens in the granulosa cells.[17]

There are two general metabolic pathways which can lead to androgens starting from the key intermediate, pregnenolone: the Δ^5 (5-ene-3β-hydroxy) and the Δ^4 (4-ene-3-oxo) pathways (Figure 1.4). Any steroid intermediate on the Δ^5 pathway may be converted to its corresponding steroid on the Δ^4 pathway by the sequential action of two enzymes: 3β-hydroxysteroid dehydrogenase, which oxidises the 3-hydroxyl of a steroid to a ketone, and Δ^5-Δ^4-isomerases, which catalyse the migration of the double bond from C5-6 to C4-5.[16] Both pathways involve a series of enzymatic reactions, eventually yielding two androgen precursors which can be readily transformed into estrogens: the 19-carbon steroids androstenedione (Δ^4-dione) and testosterone (T). To a minor extent, androgens are also synthesised in the brain via the Δ^5 and Δ^4 pathways.

In the granulosa cells of the ovaries, the enzyme aromatase then converts the C-19 androgens into C-18 estrogens, introducing two key structural modifications: the loss of the angular C-19 and the aromatisation of the A-ring. There is evidence that the adrenal cortex can also generate small amounts of E1 from its androgen precursor, androstenedione. During pregnancy, aromatisation takes place predominantly in the placenta, and most of the E1 produced is converted into estriol (E3), the active estrogen of the gestation.

In postmenopausal women, the production of estrogens by the ovaries ceases and the aromatisation of adrenal androgens represents the main source of E1 and E2. This takes place extraglandularly, in non ovarian tissues, such as fat and muscles.[18]

1.2.2 Regulation of production

Steroid-producing cells store a limited supply of hormone precursor but none of the mature, active hormone. When stimulated, the cells convert the precursor to the active hormone, which then diffuses across the plasma membrane into the blood. This is the case for estrogens, whose production is regulated by the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Figure 1.5). The gonadotropins, which are released by the anterior pituitary, do not have any direct action on bodily functions except through their specific action on the pituitary and the ovaries.[16]

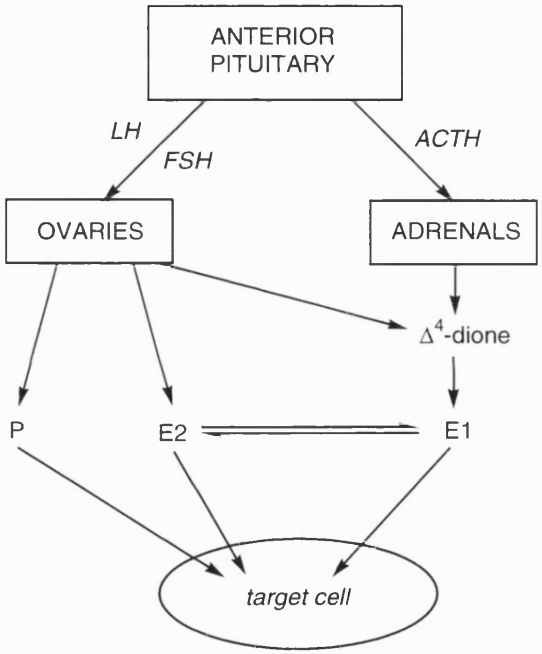


Figure 1.5 Estrogens production in the female: in premenopausal women, ovaries are the main source of estrogens while in postmenopausal women, estrogens are produced extraglandularly from the adrenals. LH, luteinizing hormone; FSH, follicle stimulating hormone; ACTH, adrenocorticotropin hormone

In premenopausal women, estrogen production follows monthly cycles during which an ovum is released. Each cycle is divided into follicular and luteal phases which correspond to pre- and postovulatory phases.[19] During the follicular phase, the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of LH and FSH, which in turn stimulates ovarian estrogen

production and induces endometrial proliferation. As estrogen levels peak, FSH secretion is inhibited and a positive feedback loop is activated, where the release of LH from the anterior pituitary is stimulated by estrogens. This results in a surge of LH which induces ovulation and marks the passage into the luteal phase of the cycle. The empty follicle produced evolves into what is called the corpus luteum, which secretes both E2 and progesterone. As the level of both hormones rises, the secretion of LH and FSH is inhibited through negative feedback. Without continued stimulation by LH, the corpus luteum regresses and the secretion of progesterone and E2 declines. This consequently stimulates the secretion of LH and FSH and the beginning of a new cycle.

In postmenopausal women, the ovaries cease producing estrogens. This is partly compensated for by production of adrenal androgens under the influence of the adrenocorticotrophin hormone (ACTH) (Figure 1.5). Without estrogen feedback, the circulating levels of FSH and LH rise substantially, which translates into a number of symptoms characteristic of the menopause. In the long term, low circulating estrogen levels are linked to a higher risk of cardiovascular diseases and osteoporosis. Estrogen replacement therapy is generally prescribed in order to prevent and relieve these symptoms.

1.2.3 Estrogen receptors and model of action

i) Early experiments and the 'two-step' mechanism

In the 1960s, thanks to the pioneering work of Jensen's and Gorski's groups, the first theory with regards to the mechanism of action of steroid hormones at the target cell emerged. Their findings converged towards the hypothesis that steroid hormones act at the level of nuclear DNA to regulate gene expression.[20,21]

Initial progress was made when *in vivo* experiments on rodents led to the distinction between target and non target tissues. After injection of ^{3}H-E2 into immature rats, it was noted that uterus and vagina would continue to incorporate and retain the radioactive hormone longer than muscle, blood, kidney and liver, where the levels

dropped relatively quickly. Further work showed that within a short time after administration, E2 was found to be concentrated in the nuclei of the target tissue cells but no nuclear uptake of the steroid was observed in the absence of cytosol.[22] Additionally, sedimentation experiments showed that ^{3}H-E2-protein complexes extracted from the cytosol and the nuclei had different sucrose density gradients, suggesting a transformation in the cytoplasmic receptor complex before entry into the nucleus.[23] Finally, the binding of these receptors to E2 was found to be specific. From these results, a two step model of action which should be applicable to all steroid hormone-cell interaction was postulated: *(a)* hormones enter the cytoplasm of the target cell and bind to a receptor for which they are specific - this may lead to alteration in the structure of the receptor; *(b)* the hormone-receptor complex diffuses into the nucleus, where it exerts its effects.

ii) Refining the model

With the identification of an estrogen receptor (ER), capable of binding E2 with high affinity and specificity in target cells, refined models were proposed to explain estrogen mechanism of action.[21] The ER, initially assumed to be a cytoplasmic protein was later found to be a nuclear receptor, as observations suggested that much of the ER may already be present in the nucleus before exposure to the hormones.[24] The cloning of the ER gene by Green *et al.*[24,25] led to the identification of the properties and the location of the functional domains of the ER protein.

The ER belongs to a superfamily of ligand-activated transcription factors, known as the steroid hormone nuclear receptor family, that can initiate or enhance the transcription of genes containing specific hormone response elements.[26] These nuclear receptors all share the same structural features: they are located predominantly in the nucleus and function as homo- or heterodimers. Biochemical and mutational analyses have indicated that the ER gene can be subdivided into several functional domains labelled A to F. The most important regions are C and E which are highly conserved and contain the DNA and hormone binding domain, DBD and HBD respectively.[27]

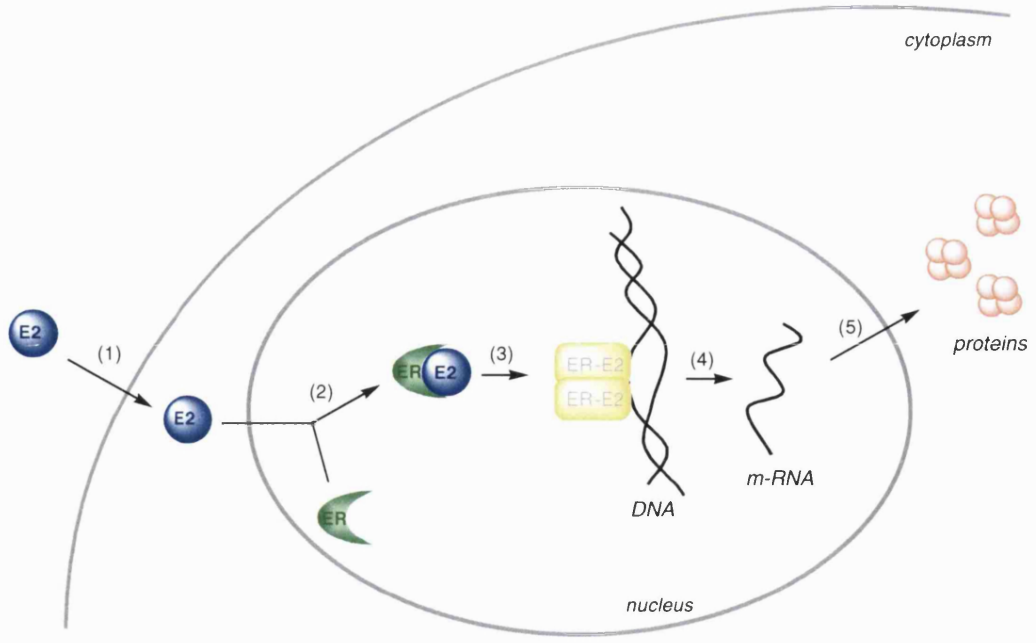


Figure 1.6 Schematic representation of the ER and its mode of action: (1) diffusion of the steroid (E2) through the cellular membrane; (2) formation of the ER-steroid complex (ER-E2); (3) conformational changes and dimerisation of the complex followed by binding to EREs; (4) transcription of a regulatory gene; (5) production of regulatory proteins.

Tissue localisation studies have shown that the ER was located in the hypothalamic-pituitary axis, liver, cardiovascular system, bone cells, uterus, vagina and in some breast cancers. In these target organs and tissues, the ER is the key mediator of estrogen action, which generally affects the growth and differentiation of target cells.[28] When an estrogen binds, it induces a conformational change within the ER which allows dimerisation of the receptor monomers.[29] The homodimer subsequently binds with high affinity to DNA-binding sites, the estrogen response elements (EREs) located in the transcriptional control regions of target genes. The DNA bound receptor activates (up-regulation) or represses (downregulation) the expression of the target gene, leading to the subsequent tissue response. A schematic representation of these events is depicted in Figure 1.6.

iii) A second estrogen receptor

Since the identification and, in the late 1980s, the cloning of the ER, it had been accepted that only one ER gene existed. Yet, nearly two decades later, a novel ER, the ERβ subtype, was cloned from a rat prostate cDNA library;[30] this was closely followed by the sequencing of its homologs in human.[31] A comparison of the amino acid sequence of the rat ERβ, with that of the first ER cloned, now referred to as ERα, showed a high degree of conservation of the DBD (96%) and HBD (58%). Similarly to ERα, ERβ displays a high affinity and specific binding of E2, and is able to stimulate the transcription of an ER target gene in an E2-dependent manner.[30,32] ERα and ERβ have an overlapping but non-identical tissue distribution and ERβ is the predominant subtype expressed in ovary, prostate, testis, spleen, lung, hypothalamus and thymus.[33,34]

The recent discovery of this new isoform of the ER has added a further level of complexity to understanding the mode of action of ERs. There is a need to distinguish ERα from ERβ pathways and there are many questions concerning how each isoform modulates estrogen action and work to stimulate growth responses, when both receptors are present in a target cell.[32]

1.3 Role of estrogens in the aetiology of breast cancer

The influence of estrogens on the development of breast cancer has long been recognised. As early as 1896, at a time when the concept of the hormone had not yet been developed, Beatson observed that advanced breast cancer could be induced to regress in premenopausal women by removing the ovaries.[35] Later on, Lacassagne was the first to indicate that a correlation probably existed between hormones and the development of cancer since the administration of estrogen could induce mammary cancer in rodents.[36] However, the precise mechanism of estrogen action at the cellular level and the influence of such hormones in carcinogenesis was only elucidated, at least partially, with the discovery of the ER and its subsequent isolation

in some breast cancer cells. By ER, we will be referring to ERα unless otherwise specified.

1.3.1 Molecular basis of estrogen action

i) Agonism and antagonism: some definitions

The ER is capable of binding a diverse set of ligands, that are broadly categorised as *agonists* or *antagonists* depending on their ability to induce or inhibit a transcriptional response. Agonists can bind the ER with different affinities, which, in the case of estrogens is correlated with their biological potency or *estrogenicity.* Additionally, the binding affinity of different estrogens to the ER parallels their ability to stimulate uterine growth and the uterine weight assay is a common way to asses the estrogenicity of a compound *in vivo.* Of the three major naturally occurring estrogens (E2, E3 and E1), E2, the most abundant estrogen released from the ovaries, is also the most biologically potent. The ability of a given compound to induce uterotrophic response is generally compared to that of E2. Intriguingly, estrogenic activity is not restricted to the steroid structure and compounds such as the synthetic estrogen diethylstilbesterol or the phytoestrogen coumestrol have a potent estrogenic activity.[37]

Compounds which antagonise the action of E2 are called *antiestrogens.* They are able to bind the ER competitively with E2 and with the same affinity, but do not elicit estrogen-related effects such as uterotrophic growth. These compounds represent an important class of drugs which aim at preventing endogenous E2 from exerting its effects on target cells, by blocking its access to the ER.

Some compounds also possess a *mixed agonist/antagonist* profile: they manifest ER agonist activity in some tissues, but oppose estrogen action in others. The properties of such compounds, called selective estrogen receptor modulators (SERMs), are exemplified by Tamoxifen. This drug, which was developed some 30 years ago and is currently used as a first line treatment for HDBC, was initially believed to act as an antiestrogen. It was subsequently shown to have estrogen-like effects on bone and

lipid metabolism (lower serum lipids) while remaining an estrogen antagonist in breast tissue.[38,39] The potentially beneficial effects of partial ER agonists in postmenopausal women, i.e. prevention of bone mass loss and reduction in cardiac diseases, led to the development of more SERMs to be used as hormone replacement therapy (HRT).

ii) Structural basis of agonism and antagonism

The cloning of the ER, followed by the determination of the crystal structure of its ligand binding domain (LBD) in complex with several molecules, have greatly contributed to the understanding of the mechanism of agonism and antagonism at the ER level.[40]

The crystal structure of the ERα-LBD in complex with either E2,[41] the synthetic non steroidal estrogen diethylstilbesterol,[42] and the antagonist raloxifene[41] has been determined. No crystal structure of the ERβ-LBD has been resolved with a natural or synthetic estrogen. It has however been determined in presence of the partial agonist genistein[43] The data collected showed that the ability of ER ligands to act as agonist or antagonist can be related to their binding mode in the vicinity of the LBD. For each ligand, the receptor forms a dimer and, although agonist and antagonist bind at the same site within the hydrophobic core of LBD, the binding modes are dramatically different for the two classes of compounds.[40,41] In fact, each class of ER ligand induces a distinctive orientation of the LBD's carboxy terminal helix, helix 12 (H12). In the presence of agonists, H12 is oriented across the cavity so that the bound ligand is completely buried within the core of LBD. In contrast, the large side-chain substituent of tamoxifen cannot be accommodated within the binding cavity. Instead, the substituent protrudes from this cavity and prevents the correct alignment of H12.[40,41] This is also the case for the full antagonist ICI 164,384, where the ligand side-chain induces a displacement of H12 and a consequent disordered conformation of the LBD.

1.3.2 Endogenous estrogens and risk of breast cancer

Many well recognised risk factors for breast cancer are estrogen-related and assert the involvement of the female sex hormone in carcinogenesis.[44,45] An early onset of menstruations (women less than 12 years old) or a late menopause (women older than 55) increase the risk of breast cancer, suggesting that the duration of exposure to ovarian estrogens is an important factor. This view is corroborated by the fact that an early menopause or removal of the ovaries early in life decreases the risk. In postmenopausal women, high estrogen levels have been correlated with an increased relative risk of breast cancer.[44]

Mutations in high risk genes such as BRCA1,[46] BRCA2 and p53 have also been correlated with an increased risk of breast cancer. These tumour suppressor genes are involved in repairing oxidative DNA damage and help maintain the integrity of the genetic material. They also play a role in cell differentiation and apoptosis in normal cells. In particular, mutations in the p53 gene have been associated with poor prognosis in breast cancer patients.[47] Somatic mutations in BRCA1 are extremely rare and the mutant gene appears to cause breast cancer only when present in the germ line, accounting for the higher risk of the disease for women who have a history breast cancer in their family. It is estimated that 70% of the mutation carriers will develop breast cancer by the age of 70.

Additional factors may also contribute to individual variation in exposure to estrogen. Obese postmenopausal women have a 2-fold increased risk of breast cancer[48] as a result of an increase in bioavailable E2. Although no correlation between E2 metabolism and nutrition has yet been strongly supported, the current hypothesis is that alcohol may affect the risk of breast cancer by increasing the endogenous E2 level.[49]

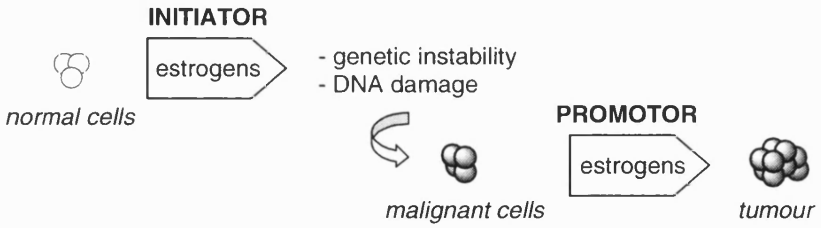


Figure 1.7 Schematic representations of the mechanisms by which estrogens increase the risk of breast cancer.

Although the precise role of estrogens in the genesis of breast cancer is unclear, several hypotheses have been put forward in an attempt to explain how these hormones exert their effects (Figure 1.7).[46,50] Estrogens are generally believed to act as *promotors* of cancer, since they are able to stimulate the growth and development of existing malignant cells in the breast. However, they may also act as *initiators*, by promoting cell proliferation in breast tissue and subsequent genetic instability. The potential genotoxicity of estrogens and some of its metabolites can also affect breast cancer risk. For instance, the catechol 4-hydroxyestradiol is highly estrogenic and might cause direct and indirect DNA damage through the formation of free radicals.[51]

1.3.3 Exogenous estrogens and risk of breast cancer

The effect of exogenous estrogens such as oral contraceptives or HRT on the risk of breast cancer has attracted much public concern. Although this topic will not be discussed in detail, it is interesting to note that in a recent study, the use of oral contraceptives was not associated with a significant increased risk among women aged 35 to 64.[52] In contrast, the results of a cohort study (the 'Million Women Study') established a relationship between the use of HRT and a higher breast cancer incidence and mortality.[53]

1.3.4 ER and breast cancer

ERs are present in both normal and tumour breast tissues as well as in estrogen-dependent and independent cell lines. ER levels vary from one woman to another, however, high levels have been directly correlated with an increased risk of breast cancer.[54]

A patient with high concentration of the receptor protein (at least 10 fmol ER per mg cytosol protein) is designated as being ER positive (ER+), while one with less than this value is regarded as ER poor or ER negative (ER-).[55,56] More than 70% of primary breast cancers in women are ER (i.e. ERα) positive and show estrogen dependent growth. It is estimated that up to 60% of ER+ breast cancers will respond to endocrine therapy (vide infra) while only 10% of ER- tumours will show a response. The assessment of the ER status is currently used to select patients for hormone-related treatments, although other biological factors may correlate with therapeutic response.[57,58]

A number of investigations have been carried out in order to establish a relation between the ER content and some intrinsic properties of tumours. Attempts to correlate ER content with tumour size, or the time of onset or incidence of distant metastases have been largely unsuccessful.[26] However, a close correlation has been observed between ER expression and the tumour growth rate: tumours with low or undetectable levels of ER proliferate more rapidly than when the ER content is higher.[54]

A correlation between the ER status of a patient and rate of recurrence of the disease also exists: women with ER+ tumours have a better prognosis with longer disease free intervals and ER- tumours tend to recur earlier and are also more aggressive.[58] Therefore the ER status, besides influencing the choice of treatment for a given patient, is a crucial prognostic indicator.

1.4 Hormone-Dependent Breast Cancer

It is now widely acknowledged that breast cancer is a hormone-dependent disease. E2 has a well established role in carcinogenesis of the breast and it is generally believed that most breast tumours rely on estrogens for their growth and development, at least in their early stage. The process which takes the disease from a cancer cell to a detectable tumour can take many years, during which time some tumours become estrogen independent. Overall, one third of all breast carcinoma remain hormone-sensitive.

1.4.1 Treatments: a brief overview

The treatment of breast cancer depends on many factors, including the characteristics of the tumour, its stage at diagnosis, its sensitivity to hormonal treatment or chemotherapy and the age and menopausal status of the patient. An early detection of breast cancer is critical to effective disease management as treatment options are greater for primary tumours. Campaigns to promote routine mammography and breast screening in women aged 50 years and older are aiming to reduce the overall mortality associated to the disease.[59]

i) Primary breast cancer

In the earliest stages of breast cancer, surgery is the treatment of choice. In most of the cases, breast conserving surgical techniques, such as local excision of lump(s) in the breast(s) (lumpectomy), are employed rather than mastectomy. To prevent any recurrence of the disease, adjuvant radiotherapy is often prescribed, particularly if breast conservation surgery has been involved.[59] There is generally no need for major surgery in patients with primary breast cancer, and in a recent trial comparing women undergoing mastectomy versus breast conservative surgery plus radiotherapy, no significant difference was observed in the overall 10-year survival.[60] Radiotherapy is also used to reduce large tumours to an operable size so that conservational surgery can be carried out.

Adjuvant hormone therapy[57] or chemotherapy, or a combination of both, can also be used to eliminate and prevent the recurrence of micrometastases after surgery. Chemotherapy is generally given to premenopausal women, while tamoxifen is the hormonal treatment of choice for postmenopausal women and ER+ patients of any age. Overall, the risk of recurrence can be decreased by 30% with adjuvant therapy.[55,56]

ii) Secondary breast cancer

For advanced breast cancers, when the tumour has spread or recurred, the aim in the treatment is no longer to cure, but to reach a palliative control. This is the case when metastases of the tumour have reached the bone, skin, lymph node or the brain.

Temporary regression of the lesions can be achieved by the use of endocrine therapy or chemotherapy and some treatments can produce a worthwhile symptom-free extension of the patient's life. The treatment strongly depends on the hormonal and ER status of the patient and the prognosis is determined primarily by the ER status.

Endocrine ablation is a treatment alternative, generally recommended in premenopausal women, whose tumour show estrogen dependency. Chemical ablation, with the use of luteinising hormone-releasing hormone (LHRH) analogues which act as pituitary downregulators, is generally given since their action is potentially reversible, unlike radiotherapy or surgical removal of the ovaries.[57,61]

1.4.2 Endocrine therapy: one answer to HDBC

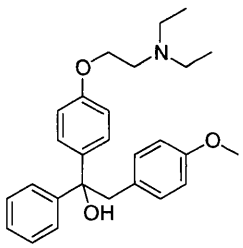
Endocrine or hormone therapy is characterised by the use of drugs specifically designed to deprive tumour cells of estrogenic stimulation, by either antagonising estrogen action at the receptor, or by blocking estrogen production pathways.

Endocrine therapy is particularly effective in women who suffer from HDBC, i.e. half of premenopausal women and two-thirds of postmenopausal women with breast cancer. Paradoxically, the incidence of HDBC is higher in postmenopausal women,

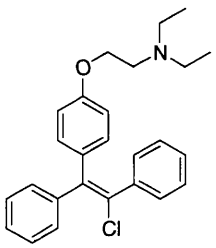
where circulating estrogen levels are relatively low, with plasma levels of E2 5- to 60-fold lower than in premenopausal women. In fact, estrogen concentration in tumour tissues is much higher than in the plasma, suggesting that tissue levels *do not* reflect circulating hormone levels.[62] *In situ* production of E1 and E2 from inactive precursors is a possible explanation for this tissue:plasma gradient.[63] Inhibition of the enzymes involved in the synthesis of active estrogenic steroids therefore represents an attractive approach for the treatment of HDBC.[64]

i) Antiestrogens

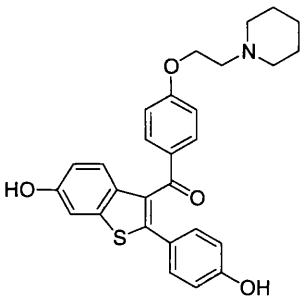
The treatment of estrogen-dependent breast cancer by antiestrogen therapy has been the front line defence against the disease for the past 30 years. Antiestrogens are able to bind competitively to the ER, thus preventing access of endogenous estrogens to their specific binding site. Since they induce a different conformation in the ER to that of agonists, their binding is normally not associated with estrogen-related biological effects.[65,66]



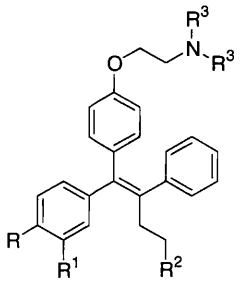
MER 25



Clomiphene



Raloxifene



Name	R	R^1	R^2	R^3
Tamoxifen	H	H	H	CH_3
4-Hydroxytamoxifen	OH	H	H	CH_3
Toremifene	H	H	Cl	CH_3
Droloxifene	H	H	OH	CH_3
Idoxifene	I	H	H	$-(CH_2)_4-$

Figure 1.8 Structure of non steroidal antiestrogens and SERMs.

Antiestrogens were initially developed in the late 1950s, as a result of a search for orally effective non-steroidal contraceptives. The first non-steroidal antiestrogen MER 25 (Figure 1.8) led to the discovery of compounds such as Clomiphene and Tamoxifen, both sharing a triphenylethylene skeleton.[39] While Clomiphene is now marketed for treating infertility, Tamoxifen has become the endocrine treatment of choice for all stages of breast cancer[39,55,56] offering a safe and simple alternative to ablative surgery, reducing the risks of recurrence and prolonging the survival of women affected by the disease. Interestingly, Tamoxifen itself does not bind to the ER but its metabolite, 4-Hydroxytamoxifen (Figure 1.8), has a 100-fold higher affinity for the receptor and is able to compete with E2 for binding.

Results of randomised trials of adjuvant Tamoxifen versus no Tamoxifen in women with breast cancer before recurrence have shown that the drug is optimally effective with a 5 year course treatment.[56] For women with ER+ tumours, receiving adjuvant Tamoxifen for 5 years, a 47% reduction in recurrence was observed during a 10-year follow up, with a corresponding 26% reduction in mortality. Patients with ER+ tumours did respond better to the treatment than those with ER- tumours and, overall, Tamoxifen was found to improve the 10-year survival for women with early breast cancer[55] irrespective of age, menopausal status, and whether previous chemotherapy has been administered to the patient. Today, several clinical trials are assessing the possible use of Tamoxifen as a preventive treatment for breast cancer. However, there are still some serious unanswered problems:[39,65-67]

(a) Tamoxifen is a partial agonist on the endometrium and long-term use was found to be associated with an increased incidence of endometrial carcinoma.

(b) Although 70-80% of breast cancers are ER+, only half of these will respond to tamoxifen. This phenomenon might be due to the polymorphism of the ER since the presence of ERβ can potentially allow estrogen-regulated growth, even in the presence of an antiestrogen.

(c) Over time, some tumours can become resistant to the treatment by becoming hormone-independent; in responding patients, the disease ultimately progresses to a resistant phenotype, because of the presence of an efficient growth control pathway independent of estrogen signalling.

The mixed agonist/antagonist properties of Tamoxifen prompted the development of a new type of antiestrogens, the selective estrogen receptor modulators or SERMs. These drugs, which emerged in the 1990s, combine the potentially beneficial effects of estrogens in postmenopausal women (i.e. maintain bone density and decrease circulating low-density lipids), while reducing a patient's risk of breast cancer.[65,66]

Raloxifene (Figure 1.8) was the first SERM to be used clinically on the basis that it could reduce the incidence of breast cancer as a beneficial side-effect of the prevention of osteoporosis. Results of trials (Multiple Outcome of Raloxifene Evaluation or MORE) confirmed this hypothesis, with a 70% decrease in risk of breast cancer observed after a 3-year treatment in postmenopausal women.[68] Additionally, Raloxifene was not associated with an elevated risk of endometrial cancer. Toremifene, Droloxifene and Idoxifen (Figure 1.8) are a new class of SERMs derived from Tamoxifen that posses a weaker agonist activity on the endometrium. Only Toremifene (Fareston) is available for treatment of advanced breast cancer in postmenopausal women and is currently under evaluation for adjuvant therapy.[69]

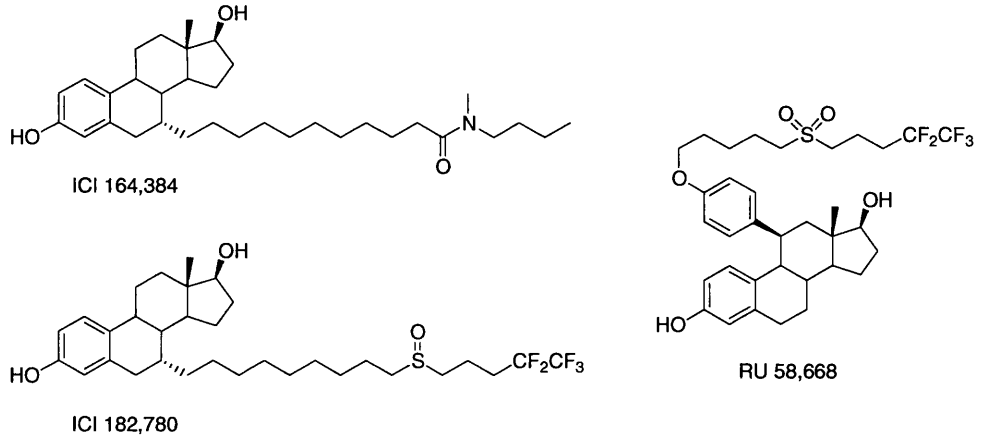


Figure 1.9 Structure of pure antiestrogens.

The search for new endocrine agents, such as pure antiestrogens, was initiated in an effort to find an alternative to Tamoxifen for patients who did not respond or developed resistance to the treatment. The first pure antiestrogen, ICI 164,384, was discovered in the late 1980s by Wakeling and Bowler.[70] This 7α-undecanamide derivative of E2 was found to be completely devoid of estrogenic activity in rat and mouse uterus, *in vitro* and *in vivo*, while retaining its affinity for the ER. The poor

bioavailability and biological profile of this compound consequently led to the design of new antiestrogens, from which ICI 182,780 (Faslodex) and RU 58,668 emerged (Figure 1.9). Similarly to ICI 164,384, they have no estrogen-like effects and can inhibit the growth of breast cancer.[71] ICI 182,780 is currently used as second-line treatment in the United States and Great Britain for advanced breast cancer, following Tamoxifen failure.[71]

Pure antiestrogens were initially believed to block dimerisation of ER complexes, consequently preventing the binding to DNA. Mechanistic investigations have however revealed that such compounds target the ER to provoke its rapid destruction, preventing tumour cell survival.

ii) Aromatase inhibitors

The use of therapeutic agents that inhibit one or several enzymes of the steroid biosynthetic pathway represents another important strategy to control the development of estrogen-dependent tumours. The enzyme aromatase, which converts androgenic C19 steroids to estrogenic C18 steroids, has been the prime target for reducing estrogen levels. This enzyme complex, which contains a cytochrome P_{450} haemoprotein, catalyses the aromatisation of the androgen A-ring with subsequent loss of the C-19 methyl group to yield estrogens.

In premenopausal women, aromatisation principally occurs in the ovaries while in postmenopausal women, estrogen synthesis from adrenal androgens takes place extraglandularly in the adipose tissue, muscle, liver and skin.[18] The aromatase enzyme is also present in more than 70% of breast tumours,[72] where estrogen levels are found to be generally higher than in normal breast tissue.[62] There is strong evidence to suggest that *in situ* aromatisation of androgens may account for the high estrogen content found within some breast tumours;[73] this is supported by the fact that 10-20 times greater levels of estrogens are found in tumour tissue vs. plasma.[62]

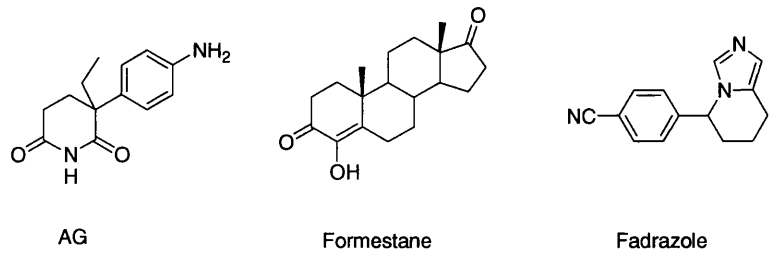


Figure 1.9 AG and second generation aromatase inhibitors Formestane (type I) and Fadrazole (type II).

Aminoglutethimide (AG, Figure 1.9) was the first aromatase inhibitor used for the treatment of breast cancer. However, its use was correlated with adrenal insufficiency and a number of undesirable side effects given its wide spectrum of action on other P_{450}-dependent enzymes.[74,75]

Attempts to improve on the original structure and increase the therapeutic index have led to a number of compounds entering clinical trials.[72,74,76] The new molecules, more selective, more active and less toxic than AG, can be classified into two types, depending on the manner in which the molecule binds to the active site. Type I compounds are steroidal, irreversible mechanism-based inhibitors derived from the natural substrate for the enzyme: androstenedione. 4-Hydroxyandrostenedione (Formestane, Figure 1.9), the first type I inhibitor to be used clinically proved to be 30-60 times more potent than AG. Its poor oral bioavailability however limited its use. Type II inhibitors are non-steroidal molecules (e.g. AG) and contain moieties such as phenylamines, imidazoles and triazoles. They act as reversible competitive inhibitors and their major interaction with the active site results from the chelation of their heteroatom(s), usually a nitrogen, to the iron heme in the enzyme, while the rest of the molecule aligns with the substrate binding domain. Fadrazole (Figure 1.9), was found to be 500-times more potent than AG *in vivo*.[76]

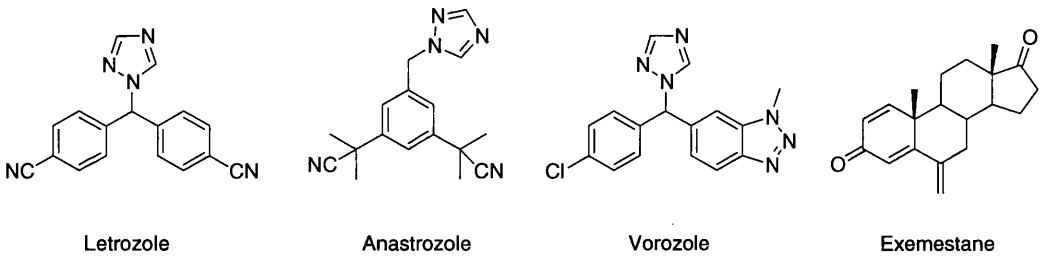


Figure 1.10 Third generation aromatase inhibitors: Letrozole, Anastrozole, Vorozole and Exemestane.

The last generation of aromatase inhibitors includes compounds such as Letrozole (Femara), Anastrozole (Arimidex) and Vorozole (Rivizor)[77] (Figure 1.10), which are type II inhibitors. These 1,2,4-triazoles are selective reversible inhibitors of aromatase: they combine high potency, being 10^3 to 10^4-fold more potent than AG, high selectivity for the enzyme and are also better tolerated. Anastrozole and letrozole are active *in vivo* and reduce serum levels of estrogens by 97% and 99% respectively. Exemestane is the latest type I inhibitor to enter clinical trials.[77]

Traditionally, aromatase inhibitors are reserved as second line treatment for advanced HDBC in postmenopausal patients whose disease is no longer controlled by tamoxifen. However, because of the extremely good toxicity profile of some of the latest aromatase inhibitors, recent clinical trials have been conducted to assess their suitability as first line treatment for HDBC. Anastrozole is one such drug, now licensed for use in the USA.

1.5 Latest strategies

In post-menopausal women, in whom breast cancer most frequently arises, peripheral and intra-tumoral synthesis of estrogens were initially considered to occur almost exclusively via the aromatase pathway. However, strong evidence has emerged over the past decade, both biochemically and clinically, that the sole inhibition of the enzyme aromatase cannot afford an effective reduction of estrogenic stimulation to tumours, the reason being that other pathways are involved in estrogen biosynthesis (Figure 1.11).

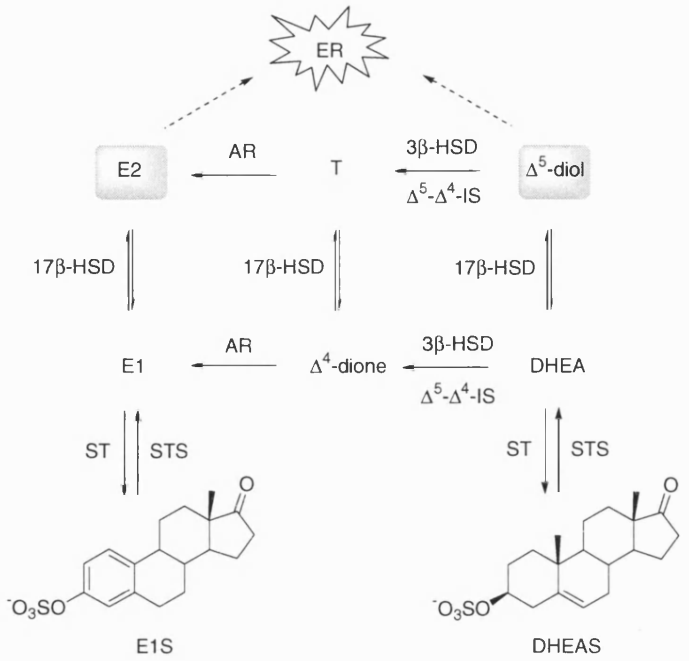


Figure 1.11 Origin of estrogens in tumour cells: the sulfatase pathway. AR, aromatase; ST: steroid sulfotransferase; STS, steroid sulfatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; Δ^5-Δ^4-IS, Δ^5-Δ^4-isomerase; ER, estrogen receptor; E1S, estrone-3-*O*-sulfate; DHEAS, dehydroepiandrosterone-3-*O*-sulfate.

The sulfatase pathway is now considered to be the major route for breast tumour estrogen synthesis since the sulfatase activity was found to provide 10-fold more E1 than the aromatase activity.[73,78] In this pathway, estrogens are synthesized from the highly available precursor E1S, *via* two enzymes (Figure 1.11): steroid sulfatase (STS) which hydrolyses E1S into E1, and 17β-hydroxysteroid dehydrogenase (17β-HSD) which reduces E1 into E2. Both the higher availability in plasma and longer half-life of E1S compared with that of unconjugated estrogens have suggested that the former may act as a reservoir for the synthesis of active steroids in postmenopausal women.[63] STS and 17β-HSD, the two enzymes responsible for the conversion of E1S into the potent estrogen E2 therefore represent valuable therapeutic targets for estrogen deprivation strategies.

1.5.1 STS inhibitors

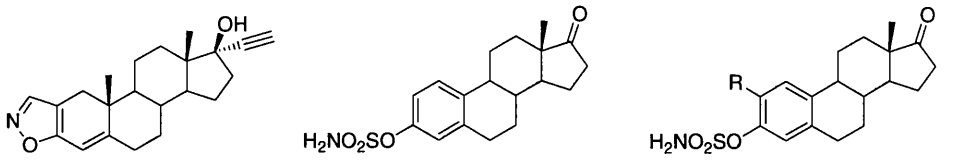
Steroid sulfatase is one of 11 sulfatase enzymes capable of cleaving sulfate esters into the corresponding alcohol or phenol. In humans, STS is a membrane bound enzyme which is responsible for the conversion of inactive 3-*O*-sulfated molecules into their corresponding potent 3-hydroxy form. The presence of STS activity has been demonstrated in human breast cancer tissue and in a number of cells lines.[79] Although STS is mainly targeted because of its pivotal role in regulating estrogen biosynthesis, it is also able to convert latent androgens into DHEA, the direct precursor of androstenediol (Δ^5-diol, Figure 1.11). Recently, this potent androgen was found to be able to bind to the ER and stimulate the growth of tumour cells *in vitro*[80] and carcinogen-induced mammary tumours *in vivo*.[81] Similarly to E2, Adiol is therefore an important metabolite for the growth and development of hormone-dependent breast tumours.[82] There is strong evidence to suggest that the sulfatase enzymes which convert E1S and DHEAS into their respective 3-hydroxy compounds are identical.[83] Therefore, as sulfated precursors can be converted into potent androgens and estrogens via one sulfatase enzyme, the development of STS inhibitors offers a viable approach for reducing estrogenic stimulation to tumours and combating HDBC.[84]

i) Steroidal inhibitors

The androgen Danazol (Figure 1.12), developed in the 1970s as a treatment for endometriosis, was the first sulfatase inhibitor to be reported.[85] Since then, several potent inhibitors have been identified for STS, some of which are progressing into clinical trials. They all share the common structural feature of an aromatic ring that mimics the phenolic A-ring of the enzyme natural substrate, E1S.

In the search for E1S analogues, a wide variety of chemical groups have been introduced at C3, of which the 3-*O*-sulfamate was found to be the most potent for the E1 skeleton.[86] The resulting compound, estrone-3-*O*-sulfamate (EMATE, Figure 1.12) inhibited STS activity with respective IC_{50}s of 80 nM and 65 pM in placental microsomes[86] and in intact MCF-7 breast cancer cells.[87] This led to the identification

of the aryl-*O*-sulfamate moiety as an active pharmacophore required for potent inhibition of STS. EMATE was also shown to inhibit steroid sulfatase activity in a time- and concentration-dependent manner[88] and was active *in vivo* on oral administration.[89] The mechanism proposed for the inactivation of the enzyme by EMATE suggests that E2 is released during the irreversible inhibition of the enzyme, which is in agreement with the later findings that EMATE was highly estrogenic in rodents.[90,91]



Danazol EMATE R = OCH_3 2-MeO-EMATE
R = OCH_2CH_3 2-Et-EMATE

Figure 1.12 Structure of Danazol, EMATE and C2 analogues of EMATE.

Most of the compounds which were subsequently developed emerged from the search for non-estrogenic inhibitors of STS. Using EMATE as a template, several modifications were introduced on the A- and D-ring, mainly at C2 and C17. Since 2-methoxy-estrogens are substantially less estrogenic than their unsubstituted parents,[92] 2-methoxy-EMATE (2-MeO-EMATE, Figure 1.12) was synthesised.[93] It was found to be a potent irreversible inhibitor of STS, although 8-times less active than EMATE in placental microsomes. This C2 analogue of EMATE was also found to possess potent anti-mitotic properties and induced tumour regression without displaying any estrogenic activity *in vivo*.[93,94] Its ethyl analogue (2-Et-EMATE, Figure 1.12), which was later developed, was as potent as the 2-methoxy analogue and had antiproliferative and antiangiogenic activity in human breast cancer cells.[95]

Molecules combining features of both STS inhibitors and antiestrogens have also been developed. Relying on the existing knowledge that an alkylamide side-chain can block the estrogen receptor activation,[70,96,97] Li *et al.* synthesised 3-*O*-sulfamates derivatives of E2 bearing long hydrophobic *N*-alkylcarbamoyl and *N*-alkanoyl side-chains at C-17.[98] The 17β-alkyl/alkanoyl group, presumably designed as membrane insert, increases the affinity of the compound for the enzyme through hydrophobic

interactions. The heptyl analogues (**1.1** and **1.2**, Figure 1.13) were found to inhibit STS with the same potency as EMATE at 10 nM, without being estrogenic at 1 μM.

1.1 R = $NHCO(CH_2)_6CH_3$
1.2 R = $CONH(CH_2)_6CH_3$

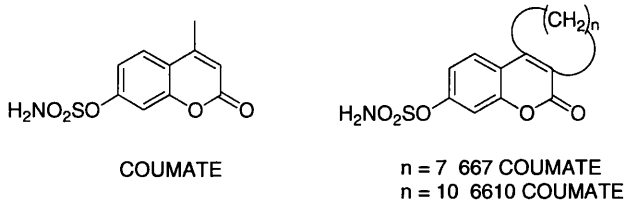
1.3 R = SO_2NH_2 R^1 = H
1.4 R = SO_2NH_2 R^1 = ^{t}Bu
1.5 R = H R^1 = H
1.6 R = H R^1 = ^{t}Bu

Figure 1.13 C-17 derivatives of EMATE: introduction of hydrophobic side-chains with α or β orientation.

Recently, the validity of targeting hydrophobic interaction at C17 of E1, E2 or EMATE was further demonstrated by Poirier and coworkers.[99-102] A series of 17α-benzyl (or *tert*-butylbenzyl) derivatives of EMATE was found to inhibit STS activity in transfected HEK-293 cells.[100] In particular, two derivatives (**1.3** and **1.4**, Figure 1.13) were highly potent, with respective IC_{50}s of 0.39 nM and 0.15 nM, being up to 14-times more potent than EMATE. These irreversible inhibitors were designed subsequent to the finding that their precursors, the corresponding D-ring derivatives of E2 (**1.5** and **1.6**, Figure 1.13), could also inhibit STS activity.[99,102] Their ability to do so without the presence of a sulfamate at the C-3 position suggests the presence of a hydrophobic pocket in the enzyme, in the region neighbouring the D-ring of these steroid substrates. Although 5-8 times less potent than EMATE, they remain the only steroidal STS inhibitors known to act via a reversible mechanism.

ii) Non steroidal inhibitors

To avoid the problems linked to an active steroid nucleus, non steroid-based inhibitors have been synthesised. Coumarin sulfamates, such as 4-methylcoumarin-7-*O*-sulfamate (COUMATE, Figure 1.14), were among the first inhibitors of that type to be identified.[87] Since the coumarin skeleton mimics the A/B ring system of estrogens, the aryl-*O*-sulfamate pharmacophore is present in coumarin sulfamates. Although COUMATE was found to be less potent than EMATE *in vitro*, it was active *in vivo* with the advantage of being non estrogenic.[103]

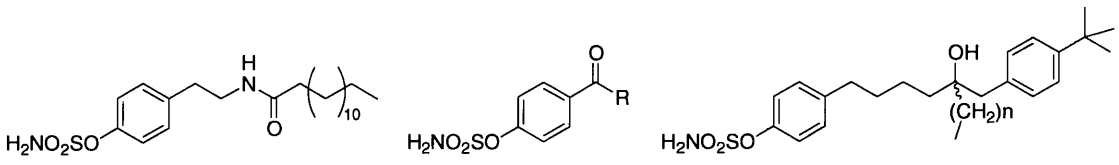


COUMATE

n = 7 667 COUMATE
n = 10 6610 COUMATE

Figure 1.14 Structure of potent non-steroidal STS inhibitors: COUMATE and tricyclic analogues.

In an attempt to improve the activity of coumarin derivatives, several modifications were considered. Increasing the hydrophobicity at C3 and/or C4 was found beneficial[103] and some tricyclic coumarin sulfamates turned out to be more potent than COUMATE, while retaining its non estrogenic characteristic.[104] Compounds bearing an extra 7- or 10-membered ring (667 and 6610 COUMATE, Figure 1.14) were the most active with respective IC_{50}s of 8 nM and 1 nM in placental microsomes. These COUMATE analogues were therefore up to 800 times more potent than COUMATE and some 25 times more potent than EMATE *in vitro*. Investigations of 667 COUMATE *in vivo* revealed that it could inhibit STS activity by 93% after administration of a single dose (10 mg/kg, p.o.) and was devoid of estrogenicity.[95] Complete recovery of STS activity occurred 7 days after drug administration.[95] This potent inhibitor also caused regression of E1S-stimulated tumour growth in a dose dependent manner[95] and is now being evaluated in a Phase I clinical trial in post menopausal women with HDBC.[84]



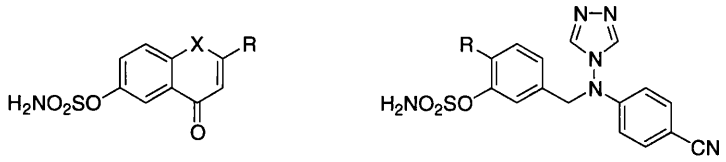
1.7 **1.8** R = C_8H_{17} **1.9**n = 6

Figure 1.15 Monoarylsulfamate-based inhibitors of STS.

In parallel to the development of coumarin-based inhibitors, Li *et al.* synthesised the first monocyclic potent STS inhibitor, *p*-*O*-sulfamoyl-*N*-tetradecanoyl tyramine (**1.7**, Figure 1.15), whose activity was found comparable to that of EMATE in placental microsomes.[105] Several compounds based on the monoarylsulfamate structure were then developed. Ahmed *et al.*[106] reported a series of irreversible inhibitors possessing a 4-sulfamated phenyl alkyl ketone structure. The best inhibitor (**1.8**, Figure 1.15)

was up to 3 times more potent than COUMATE and 7 times weaker than EMATE for the inhibition of STS in placental microsomes.[106] Additionally, a strong correlation was established between the IC_{50} of these compounds and their logP values, suggesting hydrophobicity as a crucial factor for potent inhibition.[107] Very recently, Poirier *et al.*[108] reported some 4-substituted monoarylsulfamates to be more potent than EMATE. These compounds, where the B, C and D rings of a steroid have been replaced with a mobile chain, are structurally related to the family of C17 derivatives of EMATE previously reported.[100] The optimum side-chain length was found to be of 6 carbon atoms and the resulting compound (**1.9**, Figure 1.15) was more than twice as active as EMATE against STS activity in transfected HEK-293 cells.

Non steroidal inhibitors based on the chromenone core structure have also been reported by Billich and Nussbaumer.[109,110] These compounds, which are substituted chromenone sulfamates were found to be up to 170-fold more potent than EMATE (e.g. **1.10**, Figure 1.16) against the purified human enzyme.[109] When the series was tested for their ability to induce the proliferation of estrogen-dependent MCF-7 cells, the *n*-nonyl derivative (**1.11**, Figure 1.16) was the most promising. This compound was devoid of estrogenicity at 1 μM while only 7 times less potent than EMATE.[110]



1.10 X = S, R = 1-adamantyl
1.11 X = O, R = C_9H_{19}

1.12 R = Br
1.13 R = H

Figure 1.16 Adamantyl and nonyl chromenone sulfamates (**1.10** and **1.11**) as STS inhibitors and structure of two potent DASI inhibitors (**1.12** and **1.13**).

Recently, Woo *et al.* reported the first dual aromatase-steroid sulfatase inhibitors.[111] These compounds, which are sulfamoylated derivatives of a known aromatase inhibitor, should achieve a more effective blockade of estrogen production in tumour cells by inhibiting two enzymes responsible for the biosynthesis of E1. *In vitro*, the bromo derivative (**1.12**, Figure 1.16) had IC_{50} values of 0.82 nM and 39 nM for

aromatase and STS inhibition respectively in JEG-3 cells. One dual inhibitor (**1.13**, Figure 1.16) was potent against both enzymes *in vivo*.

iii) Investigations of the catalytic mechanism for hydrolysis

Until a few months ago, the crystal structure of the human STS enzyme had not been resolved. In order to gather information on the mechanism of action of this enzyme, its active site has been extensively studied via analogy with other soluble mammalian sulfatase enzymes, arylsulfatase A (ASA) and arylsulfatase B (ASB). These enzymes, whose crystal structures are available, share extensive homology and structural similarity with the human form.[112,113] Several mechanisms for the hydrolysis of sulfate esters by sulfatase enzymes have been proposed,[113-115] from which the model for the mechanism of action of irreversible inhibitors such as EMATE was derived.[87,116,117]

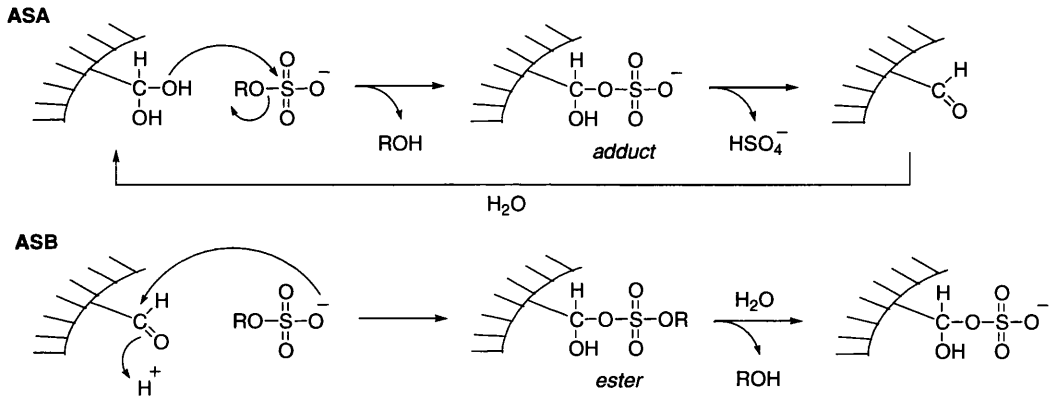


Figure 1.17 Proposed reaction scheme for sulfate ester cleavage by ASA and ASB. Adapted from Woo *et al.*[118]

Woo *et al.* recently published a review of the latest mechanistic studies on STS[118] and proposed several updated catalytic mechanisms. At the core of the catalytic cascade, a formylglycine (FGly) moiety, which results from post-translational modification of a cysteine residue of the active site, is coordinated to a metal ion, supposedly Ca^{2+}. The FGly moiety, which can be present as an aldehyde (ASB) or its hydrated form (ASA), dictates two different routes for the sulfate ester cleavage (Figure 1.17). For ASA, one of the FGly hydroxyl groups attacks the sulfur atom of the substrate, yielding the hydrolysed product and a sulfate adduct. FGly is then

regenerated after elimination of a sulfate group from the adduct. In the case of ASB, the mechanism proposed involves the nucleophilic attack of an oxygen atom of the substrate on the carbonyl group of FGly to form a sulfate ester. This is then decomposed by water into the product and a sulfate adduct.

From these studies, two mechanisms were proposed for the inhibition of STS activity by 667 COUMATE.[118] They should be applicable to any other related active site directed sulfamate inhibitor. Similarly to the hydrolysis of the substrate, these mechanisms involve a nucleophilic attack of either the hydroxyl of FGly on the sulfamoyl group or the attack of the N-atom of the sulfamate group on the carbonyl group of FGly (Figure 1.18A and 1.18B). Both mechanisms are irreversible and inactivate the enzyme. Inhibition may also occur by random specific or non-specific sulfamoylation of an essential amino-acid residue (lysine or histidine) in the STS active site.

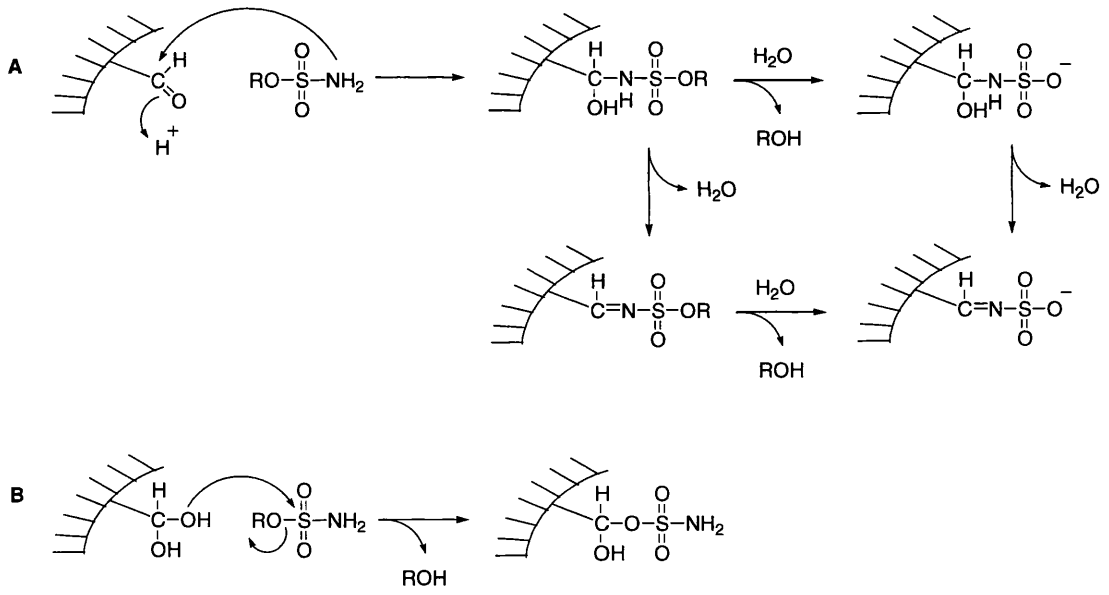


Figure 1.18 Proposed mechanisms of STS inhibition by a sulfamate inhibitor. Adapted from Woo *et al.*[118]

A recently constructed homology model of STS has offered further insight into the understanding of enzyme-inhibitor interactions.[119] Docking studies with the substrate and known STS inhibitors confirmed the predictive power of the model. These studies were however made redundant with the resolution of the crystal structure of

the human STS enzyme by Ghosh and coworkers.[120] The newly published three dimensional structure will be discussed in Chapter 2.

1.5.2 17β-HSD inhibitors

In HDBC, tumour cells possess all the enzymes necessary for the production of estrogens *in situ*, thus supporting their own growth and development. The 17β-HSD enzyme, which catalyses the final step in estrogen and androgen biosynthesis is therefore a valid target to deprive these cells of their estrogen supply.[121,122]

The human hydroxysteroid dehydrogenases, which include 3α-, 3β-, 11β-, 17β- and 20α-HSD, catalyse the stereospecific oxido-reduction reactions of alcohols or carbonyls using NADP(H) or NAD(P) as a cofactor. The 17β-HSD enzymes catalyse the reversible interconversion of the oxidised and reduced forms of steroids at the 17-position[123] and are the only reversible enzymes of the steroidogenesis. However their activity is mainly unidirectional and 17β-HSD enzymes can be classified as reductive or oxidative.[122-124]

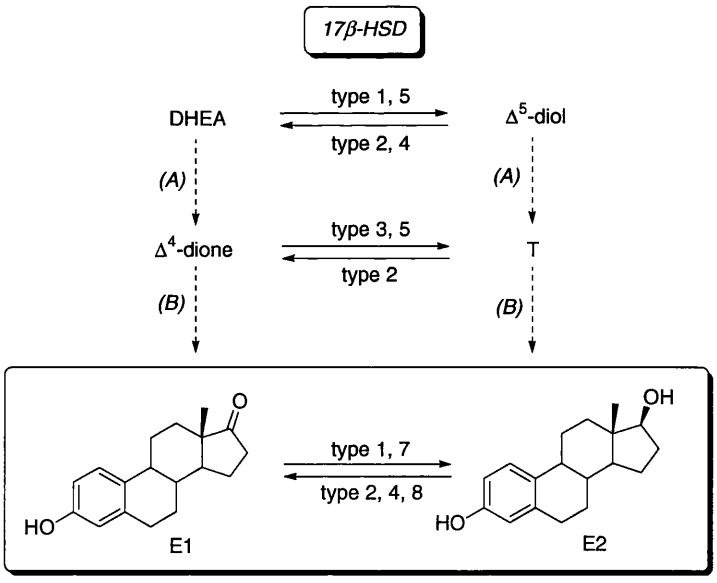


Figure 1.19 Main isotypes of 17β-HSD and the reactions they catalyse in the human steroidogenesis (unidirectional reactions shown only). (A), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerases; (B), aromatase.

17β-HSD belongs to a family of isoenzymes, 11 of which have been identified to date.[125] The cloning of the pure 17β-HSD isoenzymes has shown that eight 17β-HSD isoforms exist in humans (Figure 1.19).[126] While they all require NADP(H) or NAD(P) as a cofactor, each type has a selective substrate affinity, directional activity and a particular tissue distribution. 17β-HSD type 1, which interconverts E1 and E2, and to a minor extent DHEA and Δ^5-diol, is found in the ovaries, endometrium, placenta and in normal and cancerous breast tissue.[123] Since it preferentially reduces its substrate,[124] its activity directly supports the growth and development of estrogen-dependent tumours.[122,127,128]

17β-HSD type 1 is the best known and most studied of all the isotypes. Although the other isoforms of 17β-HSD are not discussed in detail here, it is important to note that 17β-HSD type 3 and 5 represent valid therapeutic targets for blocking the biosynthesis of testosterone and its effects on androgen sensitive diseases, such as prostate cancer. Because of the presence of several isoforms of the enzyme, it is important to achieve selectivity of drug action when targeting any 17β-HSD isoenzyme. Type 2, which is involved in the degradation and regulation of active estrogens and androgens, is more commonly used as a measure of selectivity over type 1.

Unlike STS, only few 17β-HSD type 1 inhibitors have been reported. They can be classified into irreversible and reversible inhibitors and most of the steroidal inhibitors for 17β-HSD type 1 have in common a D-ring modified structure.

i) Irreversible inhibitors

Mechanism-based inhibitors were the first irreversible inhibitors of 17β-HSD type 1 to be described in the literature.[129] These compounds, initially developed by Covey and Tobias,[130-133] are α,β-unsaturated alcohols steroidal derivatives at C20 or C17 (or pseudo C17) (Figure 1.20). Upon enzymatic oxidation, the unsaturated alcohol is converted into the corresponding α,β-unsaturated ketone (a Michael acceptor) that

can bind covalently to the enzyme through 1,4-addition of an amino-acid residue. This subsequently inactivates the enzyme.

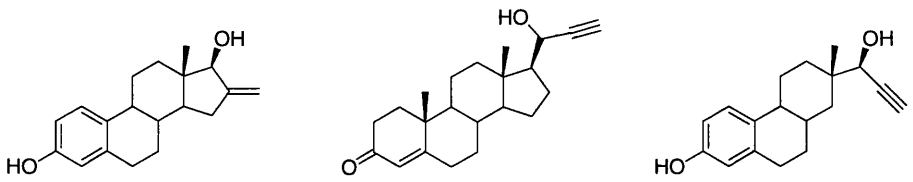


Figure 1.20 Mechanism-based inhibitors of 17β-HSD type 1.

Unfortunately, the compounds developed were either poor substrates for the enzyme or had a weak oxidation rate (or both). 16-Methylene-estradiol (**1.14**, Figure 1.20) was the most promising compound of this class, being oxidised at about the same rate as the natural substrate.[131] *In vivo*, this inhibitor was not estrogenic after a short period of 24 hours but turned out to work synergistically with E2 to produce an enhanced uterine response.[134]

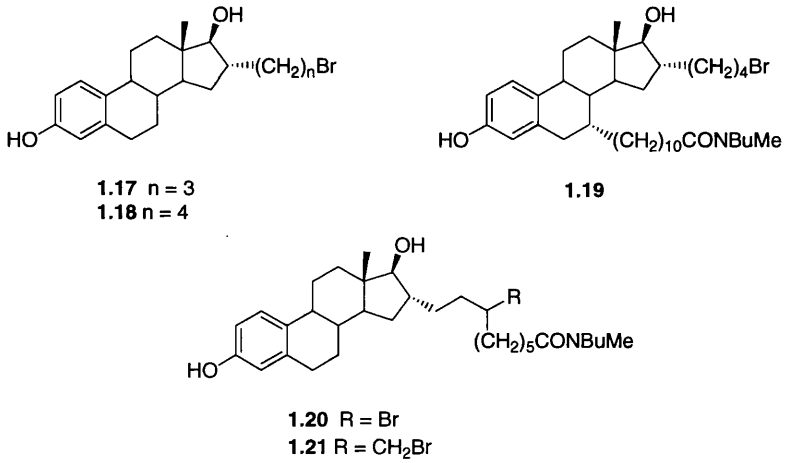


Figure 1.21 C16 derivatives of E2 as potent 17β-HSD type 1 inhibitors (**1.17** and **1.18**) and 'dual-action' inhibitors (**1.19**-**1.21**) with reduced estrogenicity.

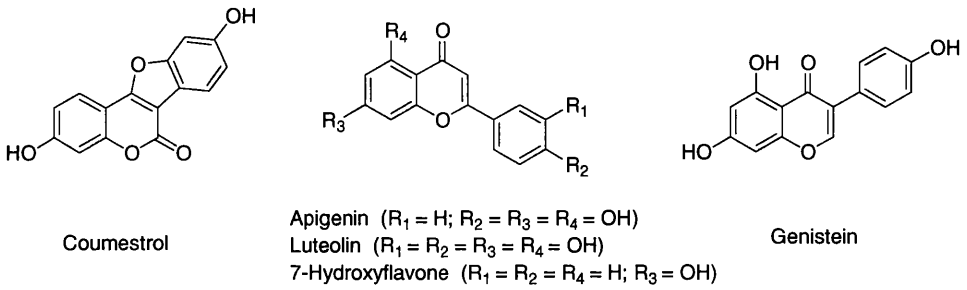
Derivatives of E2 bearing a side-chain with a good leaving group at the 16α-position have been reported as a potent class of inhibitors.[135,136] In particular, 16α-bromoalkyl-estradiol derivatives, where the side-chain exhibits reactivity towards nucleophilic amino-acid residues in the active site, were found to irreversibly inhibit 17β-HSD type 1 activity.[136-138] Compounds containing short bromoalkyl moieties at

C16 exhibited the highest activity and a comparative study revealed that 16α derivatives were more potent than their corresponding 17α analogues.[136] 16α-Bromopropyl-estradiol and 16α-bromobutyl-estradiol (**1.17** and **1.18**, Figure 1.21) were the most potent with IC_{50}s of 0.46 μM and 0.97 μM respectively in human placental cytosol. The bromopropyl analogue (**1.17**, Figure 1.21) was found to act by a competitive mechanism and inhibited enzymatic activity in a time-dependent manner.[136] However, it turned out to be a pure agonist of the estrogen receptor and stimulated the growth in the estrogen-dependent breast cancer cell line ZR-75-1.

In an attempt to eliminate the intrinsic estrogenicity of steroidal inhibitors and possibly at the same time engineer antiestrogenic properties into the molecule, an antiestrogenic C7α-alkylamide side-chain was introduced into the potent bromobutyl analogue **1.18** (Figure 1.21).[138] The resulting compound (**1.19**, Figure 1.21) was a weak inhibitor of 17β-HSD type 1, being some 40 times less potent than **1.18**. When tested on the ZR-75-1 cell line at 1 μM, **1.19** was devoid of estrogenic activity and displayed some antiestrogenic properties. Since introduction of a large side-chain at C-7α was detrimental to the activity, another approach to the design of 'dual action' inhibitors was to introduce a bromoalkylamide side-chain at the 16α position of E2.[139] The resulting compounds (**1.20** and **1.21**, Figure 1.21) had disappointing respective IC_{50}s of 14 μM and 5.7 μM for the inhibition of placental 17β-HSD type 1. However **1.20** and **1.21** were not estrogenic and displayed antiestrogenic activity at 1 μM.

ii) Reversible inhibitors

Few reversible inhibitors of 17β-HSD type 1 have been reported and most of the studies to develop such compounds have been focusing on phytoestrogens. These natural products which are structurally similar to estrogens possess a broad range of biological properties and may have a protective role against breast cancer, possibly by acting as antiestrogens. Their action on aromatase activity is well documented and in recent studies they were found to reduce the conversion of E1 into E2 catalysed by 17β-HSD type 1.[140]



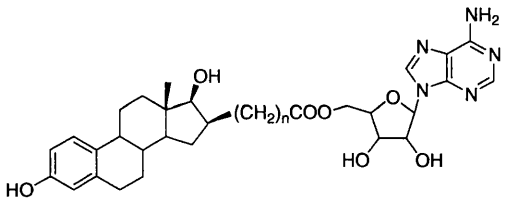
Coumestrol

Apigenin (R_1 = H; R_2 = R_3 = R_4 = OH)
Luteolin (R_1 = R_2 = R_3 = R_4 = OH)
7-Hydroxyflavone (R_1 = R_2 = R_4 = H; R_3 = OH)

Genistein

Figure 1.22 Phytoestrogens with activity against 17β-HSD type 1.

Coumestrol (Figure 1.22) was the most active of a broad range of phytoestrogens, with an IC_{50} of 0.2 μM in human placental cytosolic 17β-HSD type 1.[140] SAR studies were also carried out on flavanoids, and Apigenin (4',5,7-trihydroxyflavone, Figure 1.22) emerged as a promising compound with an IC_{50} of 0.3 μM, without being estrogenic at the inhibitory concentration.[141-143] Other natural deriatives provided interesting inhibition data such as Luteolin, 7-Hydroxyflavone and Genistein (Figure 1.23) whose respective IC_{50}s were 0.6 μM, 0.9 μM and 1.0 μM.



n = 8 EM-1745

Figure 1.23 Structure of the hybrid inhibitor EM-1745.

Recently, high affinity inhibitors of 17β-HSD type 1 in the form of E2-adenosine hybrids have been reported.[144] Both moieties (E2 and adenosine), respectively designed to interact with the substrate and cofactor binding domains, are linked by an alkyl chain tether. The optimal length of the tether was found to be of 8 methylene units (EM-1745, Figure 1.23) and the resulting compound had an IC_{50} of 52 nM in transfected HEK-293 cells. This hybrid molecule, which is the most potent 17β-HSD type 1 inhibitor to date, was also shown to act via a reversible competitive mechanism, with a Ki of 3.0 nM.

iii) Crystal structure and catalytic mechanism

The determination of the three dimensional structure of the human enzyme nearly 10 years ago has led to an atomic level description of the E2 binding pocket[145] and has shed light on its mechanism of action. More recently, crystal structures of binary complexes of 17β-HSD type 1 with E2,[146,147] C19 steroids,[147] and a dual site inhibitor[147] as well as that of a ternary complex with E2 and $NADP^+$ have been resolved.[146] The corresponding data have afforded valuable insight into the molecular determinants of substrate (estrogen) specificity[148] and have provided the basis for rational design of specific inhibitors.[149]

A Tyr-X-X-X-Lys sequence, characteristic of the enzymes of the short chain dehydrogenase family, was identified in the active site. The biochemically active form of the enzyme is a dimer and the core of the structure of each monomer is a seven-stranded parallel β-sheet surrounded by 6 parallel α-helices, three on each side of the β-sheet. E2 binds in a narrow hydrophobic tunnel, with critical residues at each end. The ternary complex E2-NADP-17β-HSD type 1 shows the presence of a triangular hydrogen-bond network between the amino acid residues Tyr155, Ser142 and O17 (Figure 1.24). In the A-ring, the 3-hydroxyl group makes specific binding interactions with His221 and Glu282. Active site-directed mutations[150] together with the construction of recombinant chimeric enzymes[151] have contributed to the identifications of the crucial residues involved in catalysis and substrate recognition. Surprisingly, the role of Glu282 could not be confirmed.[151]

The androgen Δ^4-dione is also a substrate for 17β-HSD type 1 although the enzyme has more affinity for E1. C19 steroids bind in a slightly different position to E2 in the active site to accommodate the bulk created by the extra angular methyl group, although the orientation in the active site remains similar.[147] The planar shape of the A-ring of E2, the hydrophilic interactions at C3 and C17 as well as a large hydrophobic core appear to be crucial for affinity binding in the active site.

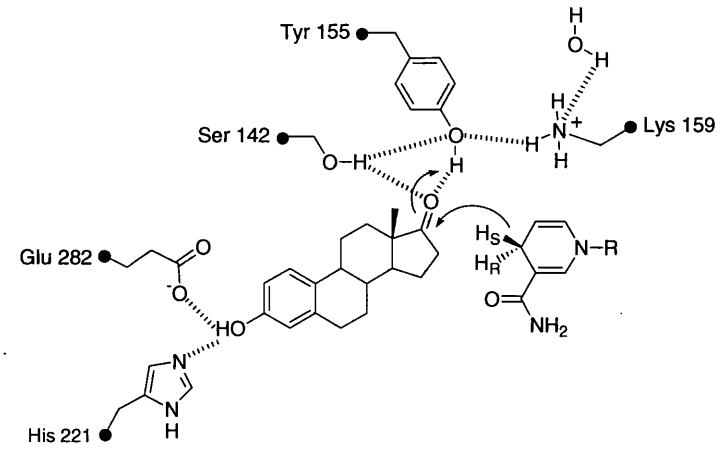


Figure 1.24 Proposed mechanism for the reduction of E1 to E2. The hydrogen bond network between the substrate and critical residues of the active site is shown. R = adenine dinucleotide.

The putative mechanism for the reduction of E1 to E2[145,146] involves the transfer of the *pro*-S hydride from the nicotinamide to the α-face of the steroid at C17, while O17 forms a strong hydrogen bond with the hydroxyl end of Tyr155 (Figure 1.24). Subsequent proton transfer from Tyr155 to E1 could be facilitated by the close proximity of protonated Lys159 and associated water molecules which lower the p*K*a of the hydroxyl proton.

1.6 Aims

Drugs that antagonise the ER or inhibit estrogen biosynthesis occupy a central role in the treatment of HDBC. Data concerning the benefits of endocrine agents such as antiestrogens and aromatase inhibitors continue to accumulate, yet there is a clear need to find alternative treatments for patients who do not respond or become resistant to these therapies. The steroidogenic enzymes STS and 17β-HSD type 1 have emerged as attractive therapeutic targets in recent years, since their inhibition can potentially achieve an efficient depletion of peripheral and local estrogen levels. This project aimed broadly to investigate the design and synthesis of a range of steroidal molecules that might possess activity against either (or both) these key enzymes.

1.6.1 Design of novel, potent, non estrogenic STS inhibitors

The development of STS inhibitors for the treatment of HDBC is still at an early stage although substantial progress has been made in recent years. At the time this project was initiated, precedent work in the area of STS inhibition suggested that:

(a) while non-steroidal STS inhibitors are based on the coumarin skeleton, most of the steroidal inhibitors result from modification of the A and/or D-ring of E1 or E2;

(b) the active pharmacophore for potent STS inhibition, an aryl-*O*-sulfamate moiety, is associated with an irreversible inhibition of the enzyme and has been incorporated in all potent inhibitors to date;

(c) hydrophobicity of the inhibitor around the steroidal D-ring or at an equivalent position is a determining factor for potency;

(d) EMATE, the landmark STS inhibitor is highly estrogenic in rodents;

(e) steroidal inhibitors are not necessarily agonists of the ER and it is possible to abolish/reduce the intrinsic estrogenicity of steroidal compounds with carefully chosen structural modifications.

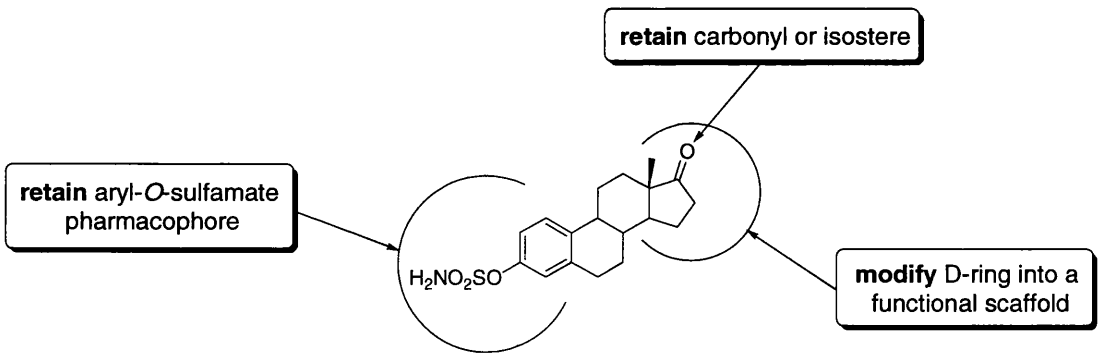


Figure 1.25 Proposed strategy to design steroidal inhibitors of STS based on EMATE.

To design potent steroidal inhibitors of STS that can be used in the treatment of HDBC, the challenge is clearly to find the best compromise between activity, estrogenicity and structural modifications on a chosen template. In a new approach to exploiting the activity of EMATE, we proposed to transform the steroidal D-ring into a functional scaffold while retaining key features, such as the aryl-*O*-sulfamate

pharmacophore and the carbonyl group at C17 (Figure 1.25). The structural modifications should allow the exploitation of hydrophobic interactions in the active site while possibly reducing the intrinsic estrogenicity of the template.

1.6.2 Design of novel, potent, non estrogenic 17β-HSD type 1 inhibitors

Inhibition of 17β-HSD type 1 is a relatively newly explored area and no pharmacophore has been identified for the generation of selective inhibitors of this enzyme isotype. The D-ring, and in particular the 16-position of the E1/E2 nucleus has emerged as a crucial location for the introduction of key functionalities. The molecular determinant responsible for high affinity binding of substrates or ligands in the active site have also been identified and have suggested the importance of hydrogen bonding at C17 and C3, as well as the presence of a planar A-ring.

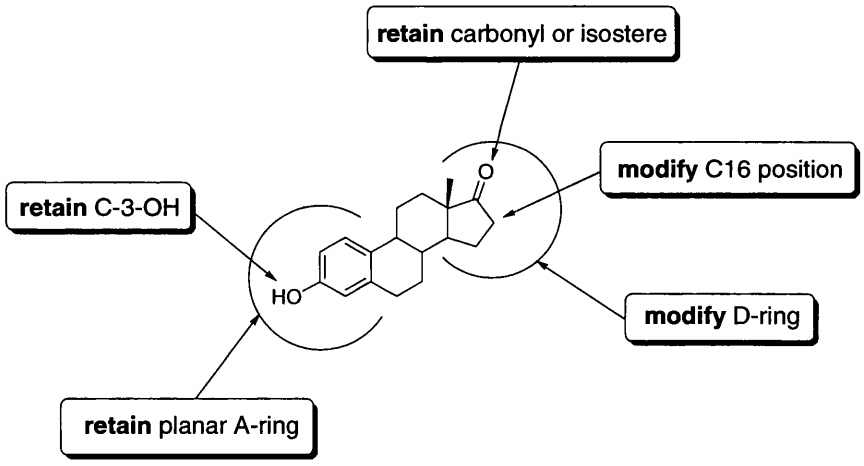


Figure 1.26 Proposed strategy to design steroidal inhibitors of 17β-HSD type 1 based on E1.

The design of novel steroidal inhibitors was therefore envisaged starting from the natural substrate for the enzyme, E1. A series of structural modifications were envisaged at C16, and more generally around the D-ring, while the essential features for binding in the active site were conserved (Figure 1.26). The synthesis of compounds having activity against 17β-HSD type 1 should contribute to extending existing SARs for estrone-based inhibitors.

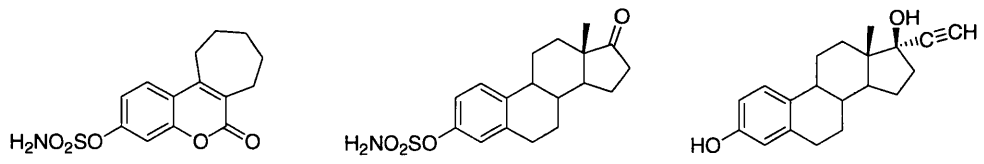
- 2 -

Steroid sulfatase inhibitors

2.1 Rationale for design

It is now acknowledged that breast tumour estrogen biosynthesis mainly occurs via the sulfatase pathway,[73,78] wherein the successive action of STS and 17β-HSD converts inactive precursors (E1S and DHEAS) into potent androgens and estrogens. STS activity therefore significantly contributes to the growth and development of estrogen-sensitive tumours and its inhibition may thus help reduce estrogen levels in patients suffering from HDBC.

Over the past 15 years several generations of STS inhibitors have been developed. The most advanced compound, 667 COUMATE (Figure 2.1), is a non steroidal drug which inhibits STS activity with an IC_{50} of 8 nM in placental microsomes.[104] It has the advantage of being active *in vivo* by oral administration and has just entered Phase I clinical trials.[84] Although several steroidal inhibitors were found to parallel the activity and properties of 667 COUMATE, no candidates could potentially be developed for the treatment of HDBC as they were also found to be agonists of the estrogen receptor. EMATE (Figure 2.1), the first highly potent steroidal inhibitor of STS to be discovered was also found to be more estrogenic than 17α-ethynylestradiol (**2.1**, Figure 2.1) on oral application in rodents.[90]



667 COUMATE EMATE **2.1**

Figure 2.1 Structure of 667 COUMATE, EMATE and 17α-ethynylestradiol **2.1**.

In order to reduce the intrinsic estrogenicity of active steroidal compounds while retaining their activity, several structural modifications have been investigated in both the E2 and EMATE series. The steroidal D-ring was mainly targeted and 16/17-substituted compounds were synthesised in order to build an SAR. Early studies on derivatives of E2 substituted at C16 indicated that the ER has a moderate tolerance for bulky substituents[152] and the introduction of 17α-alkynylamide side-chain on E2

decreased its estrogenicity.[97] More recently, a series of 17α and 17β alkylamide derivatives of E2 and EMATE proved to be less estrogenic than their parents, while retaining their STS inhibitory activity.[98,101] Moreover, hydrophobicity of the substituents at C17α was found to be crucial for powerful inhibition of the enzyme.[99,100,102] These results indicate that modifications in the D-ring region of E2/EMATE not only reduce or abolish estrogenicity, but also create some favourable interactions within the active site when the side-chain is hydrophobic or bulky. This suggests the presence of a hydrophobic pocket in the active site around the steroidal D-ring.

In order to develop non estrogenic steroidal inhibitors of STS and expand the SAR around the steroidal D-ring, we decided to design and synthesise new derivatives of EMATE, where the D-ring is structurally modified. Ideally, the target molecules should retain EMATE-like properties against STS and allow the best compromise between activity, lack of estrogenicity and accessibility.

Literature reports of structurally modified D-ring on estrogen templates mostly deal with the expansion of the 5- into a 6-membered ring, accompanied by the introduction of an heteroatom, yielding lactams and lactones.[153-157] In particular, azasteroids have been found of biological interest, with reported antibacterial[158,159] and antifungal activities. A structural modification of interest was the conversion of the cyclopentane D-ring into a piperidinedione moiety resulting in a 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivative.[156,160] Converting the D-ring of EMATE into such a moiety would therefore yield 3-sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (Figure 2.2, R = H), a molecule which can serve as a template for the design of novel STS inhibitors. This new EMATE derivative combines:

(a) structural modification of the D-ring into an imide moiety: it was envisaged that this may help reduce intrinsic estrogenicity of the molecule and enable an easy introduction of a variety of side-chains onto the D-ring;

(b) retention of EMATE-like features, namely the 3-*O*-sulfamate pharmacophore at the A-ring end and the isosteric C17 carbonyl at the D-ring end, that should ensure recognition and inhibition of STS;

(c) facility and rapidity of access from E1: according to the literature,[156,160] such D-ring modification can be achieved in two steps from E1.

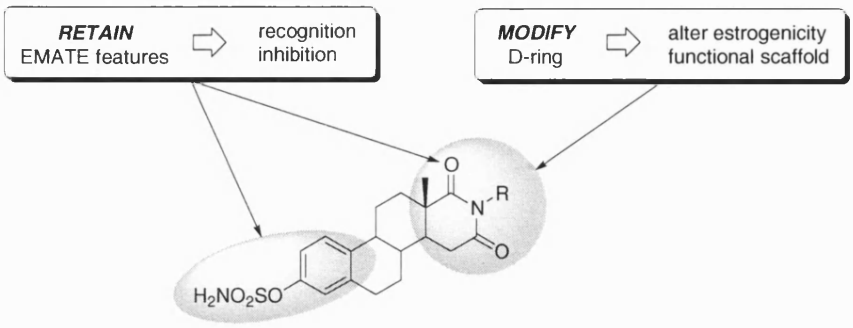


Figure 2.2 General structure for proposed STS inhibitors (R = side-chain).

It was therefore decided to synthesise a series of compounds of the above general structure (Figure 2.2), where R corresponds to side-chains designed to enhance hydrophobic interactions with the active site of STS. The combination of a D-ring modified template with bulky/hydrophobic side-chains should generate compounds devoid of estrogenic activity.

2.2 Synthesis

In order to allow versatility, the synthesis was designed around a common intermediate, from which the series of *N*-alkylated compounds was easily generated (Figure 2.3). This precursor was protected at the 3-position to avoid competing *O*-alkylation during functionalisation of the imido group. The synthesis of the target compounds therefore required *(a)* the design of a short, reliable, high yielding pathway to access the key synthetic intermediate in large quantities; *(b)* an efficient introduction of diversity to yield a series of analogues in a rapid manner. This strategy is depicted in Figure 2.3.

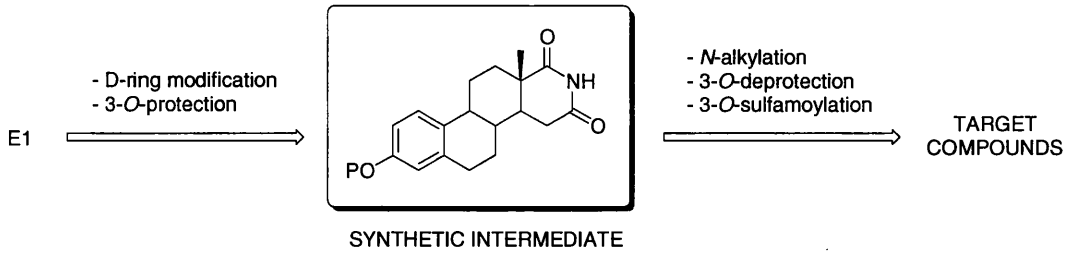


Figure 2.3 Synthetic strategy for access to target compounds (P = protecting group). The key steps are shown above the arrows.

2.2.1 Access to the synthetic intermediate

Since the discovery of E1, a number of structurally modified steroids have been developed and synthesised. Although there has been considerable interest in the synthesis of aza- and other heterocyclic steroids, few synthetic routes have been reported for the preparation of 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivatives. On the development of such structurally modified D-ring estrogens, two major synthetic approaches have been used over the years: *(a)* Beckmann rearrangement of estrone oximes; *(b)* oxidative cleavage of the D-ring with subsequent cyclisation by condensation, using a nitrogen nucleophile.

i) Beckmann rearrangement

The rearrangement of oximes into amides was first reported in steroids in 1951.[154] 17-Ketosteroid oximes were shown to rearrange into 6-membered D-ring lactams of steroids (Figure 2.4A) upon reaction with *p*-acetylaminobenzenesulfonyl chloride in pyridine.

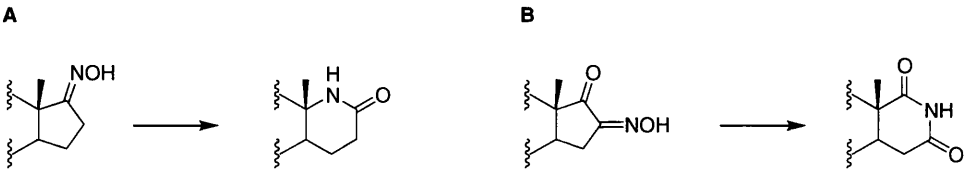
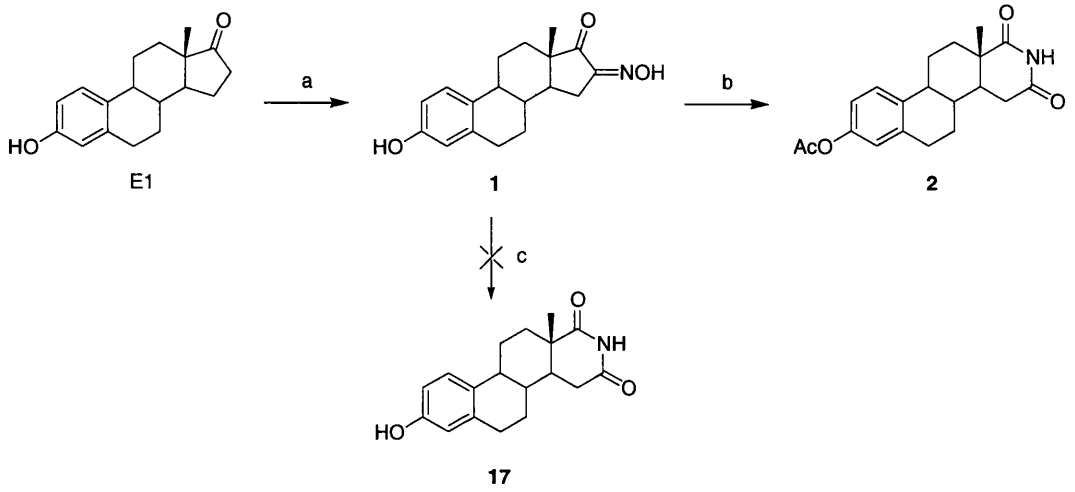


Figure 2.4 Structural modifications of the steroidal D-ring via 17- and 16-oxime derivatives (the steroidal skeleton is not shown).

Shortly afterwards 17-keto-16-oximino steroids were found to yield 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (Figure 2.4B) when reacted with an excess of thionyl chloride in benzene,[155] and 16-oximino-estrone rearranged into the 6-membered imido derivative using thionyl chloride in dioxane.[156] Recently, Gupta *et al.* reported the synthesis of such D-ring imido derivatives of E1 protected at C3 in two steps from E1,[160] which prompted us to investigate this pathway for the synthesis of our synthetic intermediate.



Scheme 2.1 Synthesis of **2** via Beckmann rearrangement. Reagents: (a) $KOC(CH_3)_3$, $(CH_3)_2CH(CH_2)_2ONO$; (b) Ac_2O/AcOH, reflux; (c) $SOCl_2$, dioxane or THF, rt or reflux.

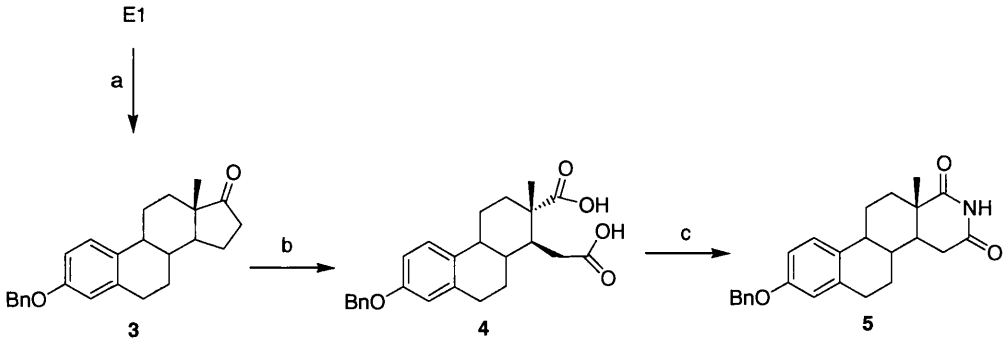
16-Oximino-estrone **1** was prepared using an adaptation of the method of Litvan and Robinson.[161] E1 was stirred with a freshly prepared solution of potassium *tert*-butoxide in *tert*-butyl alcohol and the resulting enolate reacted with excess isoamyl nitrite to give the expected oxime in 63% yield (Scheme 2.1). A Beckmann rearrangement of **1** was then carried out following the procedure of Gupta and coworkers,[160] which involves refluxing in a mixture of acetic acid and acetic anhydride. This afforded the imido derivative **2** in a 65% yield (cf. lit.[160] 53%). While the Beckmann rearrangement step afforded concomitant D-ring functionalisation and 3-*O*-protection, the overall yield of 40% from E1 for the synthesis of the intermediate **2** was only moderate. Attempts to perform the rearrangement of **1** using thionyl chloride in dioxane failed to give the expected product **17**, which contrasted with the report of Matkovics and coworkers.[156] Changing the solvent to THF, or the reaction conditions from room temperature to

reflux was equally unsuccessful. This however confirmed Kaufmann's earlier observation that the rearrangement did not proceed unless the steroidal 3-hydroxy group was esterified.[154] An alternative pathway was therefore investigated.

ii) Sequential D-ring cleavage/D-ring closure

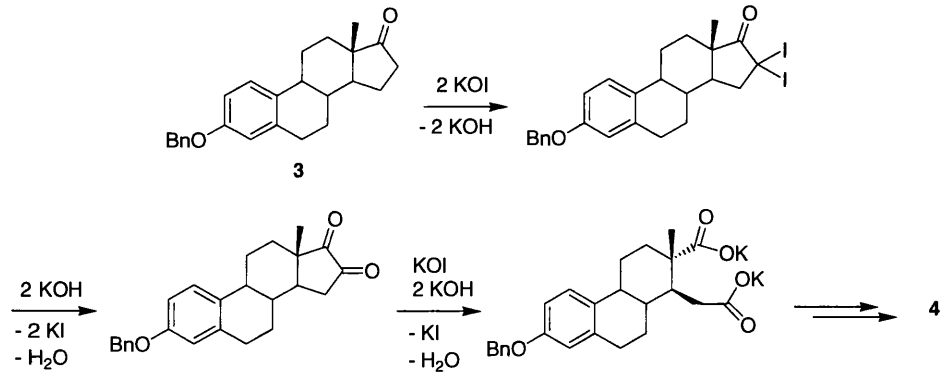
Oxidative cleavage of the D-ring of steroids has been reported as early as 1932. When E2 or E3 were fused with alkali, a mono- (doisynolic acid) and dicarboxylic acid (marrianolic acid) steroid derivative were respectively obtained.[162] Several other methods were then investigated in order to cleave the cyclopentanone ring: the D-ring of 16-ketoestradiol was opened using lead tetraacetate,[163] or via alkaline hydrolysis;[164] acidic or alkaline hydrogen peroxide cleaved the D-ring of E1 at room temperature.[153,165] In 1945, Heer and Miescher reported the direct conversion of 3-benzyl-*O*-estrone (**3**, Scheme 2.2) into 3-benzyl-*O*-marrianolic acid (**4**, Scheme 2.2) using a hypoiodite mediated oxidative cleavage.[166]

It was envisaged that the condensation of dicarboxylic compounds such as marrianolic acid or **4** with an amine would yield 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivatives. In a study on the synthesis of 16-azaestrones and 17-aza-D-homoestrones, Back et *al.* reported the reaction of a marrianolic acid derivative with liquid ammonia in methanol (100°C, pressure reactor) to yield a D-ring lactam.[157] Although, to the best of our knowledge, the conversion of carboxylic acid precursors to D-ring imides has not been reported in the literature, we decided to investigate this pathway as an alternative to the Beckmann rearrangement to access our synthetic intermediate. Since the 3-hydroxyl group needs to be protected for the second part of the synthesis, we decided to follow the exact procedure of Heer and Miescher, where the reaction is carried out on benzyl-protected E1.



Scheme 2.2 Alternative method to Beckmann rearrangement for the synthesis of D-ring modified derivatives of E1. Reagents: (a) NaH/DMF, BnBr (b) I_2, KOH, MeOH then KOH reflux; (c) urea, 180°C.

The benzyl ether **3** was prepared by an adaptation of a reported procedure,[167] by reacting E1 with NaH and benzyl bromide in DMF. Oxidative cleavage of the cyclopentanone moiety was then performed as described in the literature,[166] using an alkaline solution of potassium hypoiodite (Scheme 2.2). This reaction, which is an adaptation of the iodoform reaction (also called Lieben's test), is mostly known for its application in the characterisation of methyl carbinols and methyl ketones.[168] However, haloform reactions are also occasionally used as a synthetic method to access carboxylic acids[166] and have been performed with sodium hypobromite[169] and sodium hypochlorite.[170]



Scheme 2.3 Key steps for the iodoform reaction on **4**.

By reacting **3** with an excess of KOH and iodine, the methylene ketone function at C16 was bishalogenated, oxidised and cleaved following the steps depicted in Scheme 2.3. Since the reaction was carried out in methanol, the crude product was

refluxed in an alkaline solution to cleave any methyl ester formed as a side-product. After purifications, the dicarboxylic acid **4** was isolated with an optimised yield of 75%.

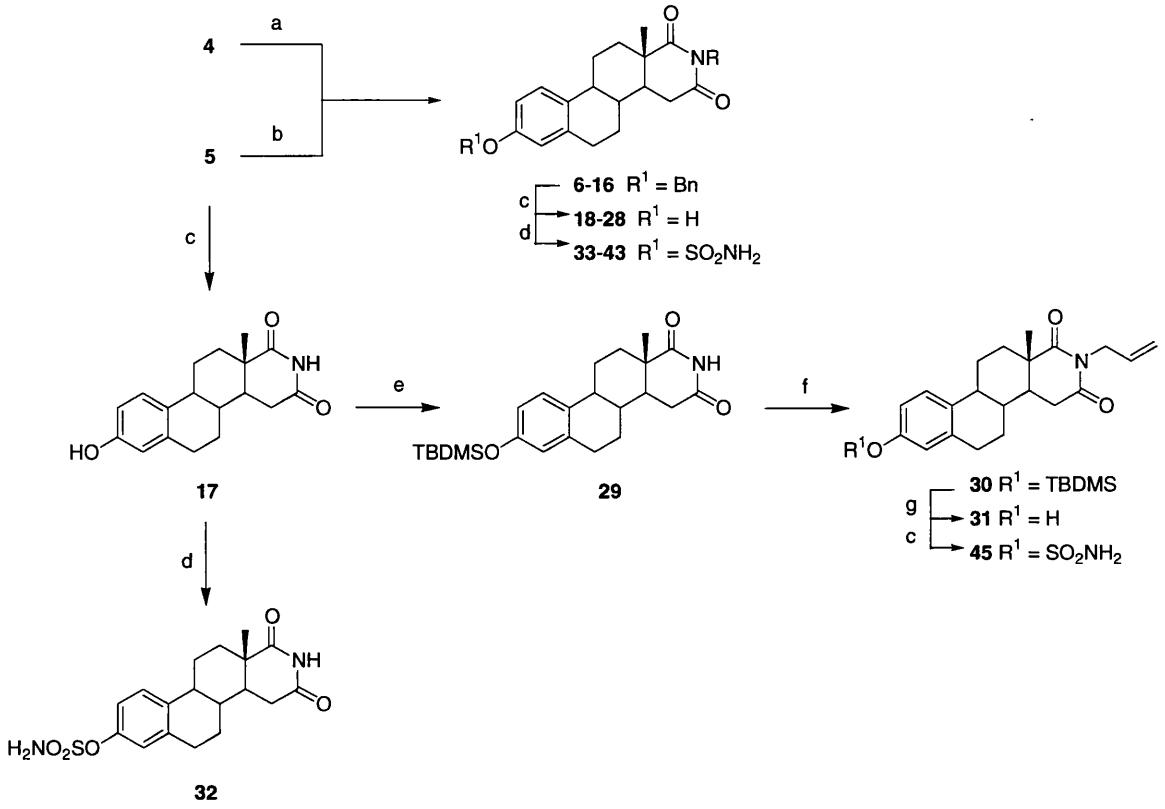
Thermal cyclisation of **4** was carried out with urea as the nitrogen source (Scheme 2.2). After briefly fusing the reagents together at 180°C, a 6-membered ring was formed from the condensation of urea and the dicarboxylic acid moiety of **4**. The synthetic intermediate **5** was obtained in high yield (80-89%), with an overall yield of 55% from E1 and its structure was assigned by analogy with that of **2**. The presence in the ^{1}H NMR spectrum of a characteristic peak for NH at δ 10.63 as well as peaks for two carbonyls at δ 172.1 and δ 178.9 in the ^{13}C NMR spectrum were indicative of the imide function. Despite the presence of an additional step, the alternative pathway to access to the intermediate **5** represents a significant improvement over the literature method for the preparation of imides derivatives of E1. Both the synthesis of **4** and **5** could be carried out on multi-gram scale, affording a generous supply of the intermediate within a few steps. Interestingly, compound **4** can also serve as a synthetic intermediate and may readily react with alkylamines to yield *N*-alkylated products (see section 2.2.2).

2.2.2 Synthesis of the target compounds

The conversion of **5** into the final compounds was easily achieved following a three-step sequence: *N*-alkylation, 3-*O*-deprotection and 3-*O*-sulfamoylation (Scheme 2.4).

The *N*-alkylated derivatives were obtained after conversion of **5** into its conjugate base and reaction with various alkyl halides. In this manner, a variety of side-chains were successfully introduced on the D-ring affording **6-16** (Table 2.1) in yields ranging from 75 to 97%. The use of an alkylation reaction to introduce diversity at this stage proved expedient due to the broad range of commercially available alkyl halides and the relative reliability and simplicity of the reaction. However, for the introduction of 1-pyridin-3-ylmethyl and benzyl side-chains, yields for the *N*-alkyl compounds were surprisingly low and the corresponding products were accessed directly from **4** (Scheme 2.4). When heating the dicarboxylic acid with 3-

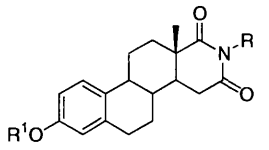
picolylamine and benzylamine, **15** and **16** were respectively obtained in 77% and 65% yields. This represents an alternative pathway for the synthesis of those derivatives where the alkyl halides are commercially unavailable or unreactive towards *N*-alkylation of **5**.



Scheme 2.4 Synthesis of the target compounds **32-45**. Reagents: (a) RNH_2, 180°C; (b) NaH/DMF, RX; (c) Pd/C, H_2, MeOH/THF; (d) $ClSO_2NH_2$/DMA or NaH/DMF, $ClSO_2NH_2$; (e) TBDMSCl/Imidazole, DMF; (f) NaH/DMF, CH_2CHCH_2Br; (g) TBAF/THF.

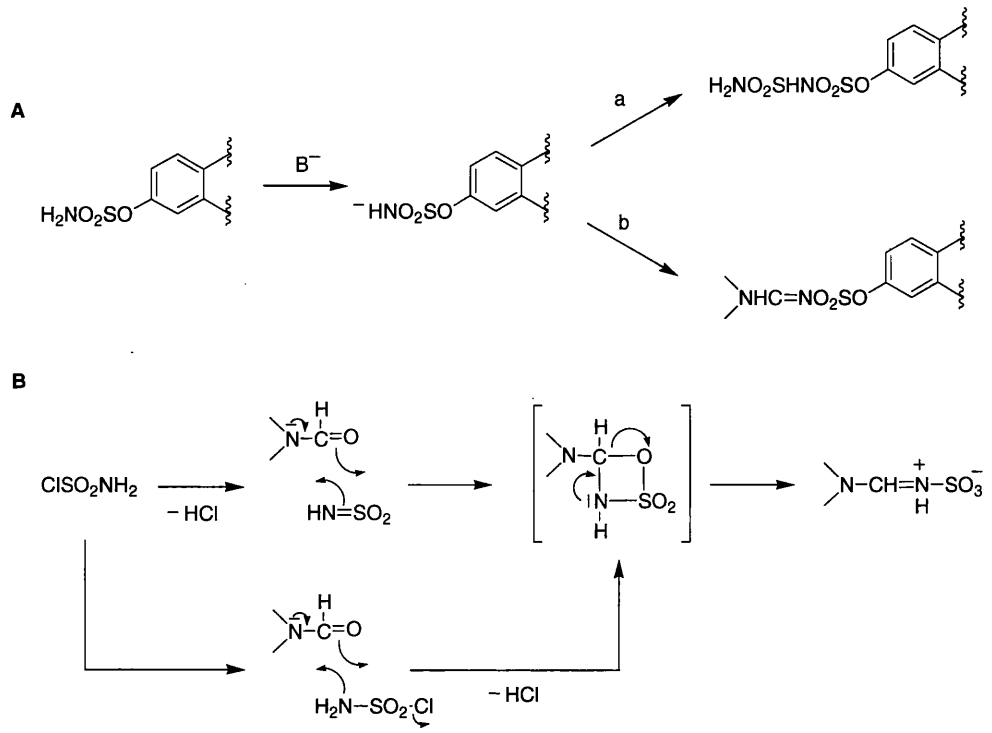
The benzyl ethers of **6-16** were then cleaved by catalytic hydrogenation using Pd/C to afford the corresponding phenol derivatives **18-28** (Table 2.1) in high yields. For the introduction of an unsaturated side-chain onto the D-ring, a different protecting group had to be used since the debenzylation step is likely to hydrogenate the unsaturated group concurrently. Compound **5** was debenzylated to give **17**, then re-protected as a *tert*-butyldimethylsilyl ether to give **29** in 78% yield (Scheme 2.4). Alkylation of **29** with allyl bromide afforded **30** in 92% yield, and subsequent deprotection using TBAF in THF gave **31**. This particular approach, which was developed for the introduction of an allyl moiety on the D-ring, should also be applicable to the introduction of other unsaturated groups.

Table 2.1 Numbering of the 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivatives synthesised.



	R^1			
R	Bn	TBDMS	H	SO_2NH_2
H	**5**	**29**	**17**	**32**
CH_3	**6**		**18**	**33**
CH_2CH_3	**7**		**19**	**34**
$(CH_2)_2CH_3$	**8**		**20**	**35**
$(CH_2)_3CH_3$	**9**		**21**	**36**
$(CH_2)_4CH_3$	**10**		**22**	**37**
$(CH_2)_5CH_3$	**11**		**23**	**38**
$(CH_2)_4Br$	**12**		**24**	**39**
CH_2cyclopropyl	**13**		**25**	**41**
CH_2Ar^tBu	**14**		**26**	**42**
CH_2pyrid-3-yl	**15**		**27**	**43**
CH_2Ar	**16**		**28**	**44**
$CH_2CH{=}CH_2$		**30**	**31**	**45**

Sulfamoylation of the phenols was then performed following a recent procedure described by Okada *et al.*[171] in which the sulfate ester of various phenols was formed in the absence of base, in the aprotic solvent *N,N*-dimethylacetamide (DMA). With the growing interest in the biological properties of sulfamic acid esters, several methods have been investigated for the synthesis of sulfamates. Howarth *et al.*[86] have reported the sulfamoylation of E1 to proceed in high yields via its sodium salt, using NaH in DMF and a large excess of sulfamoyl chloride. Esterification of E1 was reported to be equally successful in dichloromethane (DCM), using 2,6-di-*tert*-butyl-4-methylpyridine as a base.[100,172] While in our group, sulfamoylation was usually carried out using Howarth's method, the newly reported procedure was of interest for several reasons.



Scheme 2.5 A, Side-reactions occurring during sulfamoylation in DMF, in the presence of a base (B^-): (a) $ClSO_2NH_2$; (b) DMF. Only the A-ring of the steroid backbone is shown. B, Proposed mechanism for the reaction between sulfamoyl chloride and DMF.[172]

First, the absence of a base in the reaction mixture reduces the risk of forming by-products, as a result of the deprotonation of the 3-*O*-sulfamate moiety. Such nucleophilic species can readily react with either sulfamoyl chloride, yielding a sulfamoyl sulfamate, or with the solvent DMF to give an azomethine type of adduct (Scheme 2.5A).[172] Second, the competing reaction between sulfamoyl chloride and DMF (Scheme 2.5B), which results in the formation of *N*,*N*-dimethylaminomethylene sulfamic acid as a by-product,[172] does not occur in DMA.[171] This reaction, which has been reported to proceed via electrophilic addition of azasulfene to the carbonyl oxygen of DMF to yield a cyclic intermediate,[172] can conceivably take place with sulfamoyl chloride. Higher yields and shorter reaction times were reported when DMA was used as a solvent instead of DMF, and only 2 equivalents of sulfamoyl chloride were required for optimal yields. Using these conditions, the sulfamates **32-38**, **41**, **42**, **44** and **45** (Table 2.1) were obtained mostly in high yields after a short reaction time.

When **24** was subjected to Okada's sulfamoylation conditions,[171] an unexpected side-product was formed in greater amount than the anticipated sulfamate, **39**. Despite only one apparent C18-H_3 signal, the presence of two deshielded triplets around δ 3.4-3.6 in the ^1H NMR of the chromatographed mixture, as well as mass peaks of *m/z* 469.2 and 513.1 suggested that **39** (*m/z* = 513.1) and a structurally related compound were formed. The structure of the side-product **40** was found to be identical to that of **39**, except for the replacement of the bromine by a chlorine atom in the side-chain. Attempts to separate both products by flash chromatography or recrystalisation failed; however, the compounds could be separated by HPLC (Figure 2.5) and they were finally isolated using preparative HPLC. From the corresponding traces, the proportion of **40** vs. **39** was found to be 1.4:1.

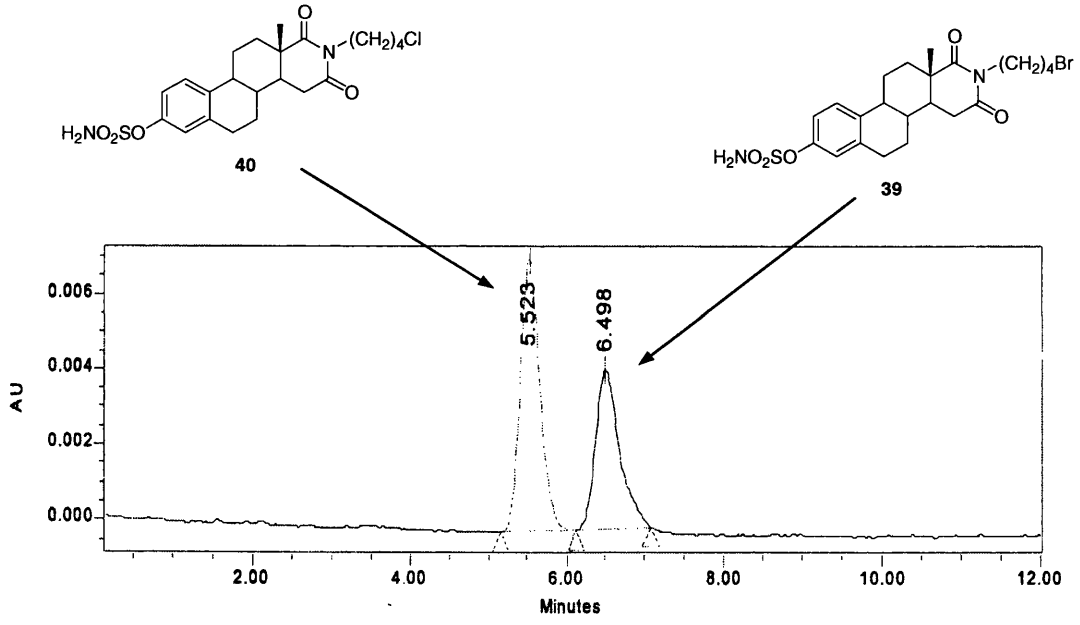


Figure 2.5 HPLC chromatogram of the mixture of **39** and **40**. Elution with MeOH/H_2O, 68:32; λ_{max} = 267.5 nm.

When the sulfamoylation of **24** was carried out using Howarth's method,[86] **39** was isolated with a yield of 81% as the sole product of the reaction. Clearly, under Okada's conditions, a halogen exchange takes place on the side-chain between the bromine and a chlorine atom. The fact that **39** only is obtained using Howarth's conditions excludes the possibility of unreacted sulfamoyl chloride being the source of chlorine for the side-reaction. Presumably, the absence of a base in Okada's

conditions leads to the formation of HCl during the sulfamoylation, whereas any free chlorine is trapped as NaCl under Howarth's conditions. The halogen exchange might therefore occur between **24** or **39** and the HCl formed as a result of the sulfamoylation reaction.

Similarly, sulfamoylation of **27** was achieved with better yields of **43** when Howarth's method was used.[86] This was consistent with the difficulties that we have observed in the past in sulfamoylating compounds possessing pyridine groups using Okada's procedure.

2.2.3 Crystal structure of compound 35

In order to elucidate the orientation of the atoms in the D-ring and in the side-chains, as well as to gain structural information for potential molecular modelling studies, the crystal structure of **35** was determined.

A crystal (approx. dimensions 0.20×0.17×0.08 mm) obtained from slow recrystalisation in acetone/hexane, was used for data collection. The ORTEX[173] plot of the asymmetric unit of **35** is shown in Figure 2.6, along with the labelling scheme used. The sulfamate group, all four rings, and the key features of the modified D-ring are clearly visible. The stereochemistry at C13 was also confirmed. All C-C bond lengths were in the range 1.375-1.545 Å and both C-N bonds of the imide group had similar lengths (1.397 Å and 1.398 Å). The bond between the imido N-atom and C1' of the propyl side chain measured 1.476 Å and the C-O bonds of the C16 and C17 carbonyl measured respectively 1.215 Å and 1.225 Å. For full details see Appendix 1.

As expected, the D-ring is in a half chair conformation since the imide functionality is planar. Molecules are hydrogen-bonded to each other via two interactions: between NH1A and O2 and between NH2A and O5.

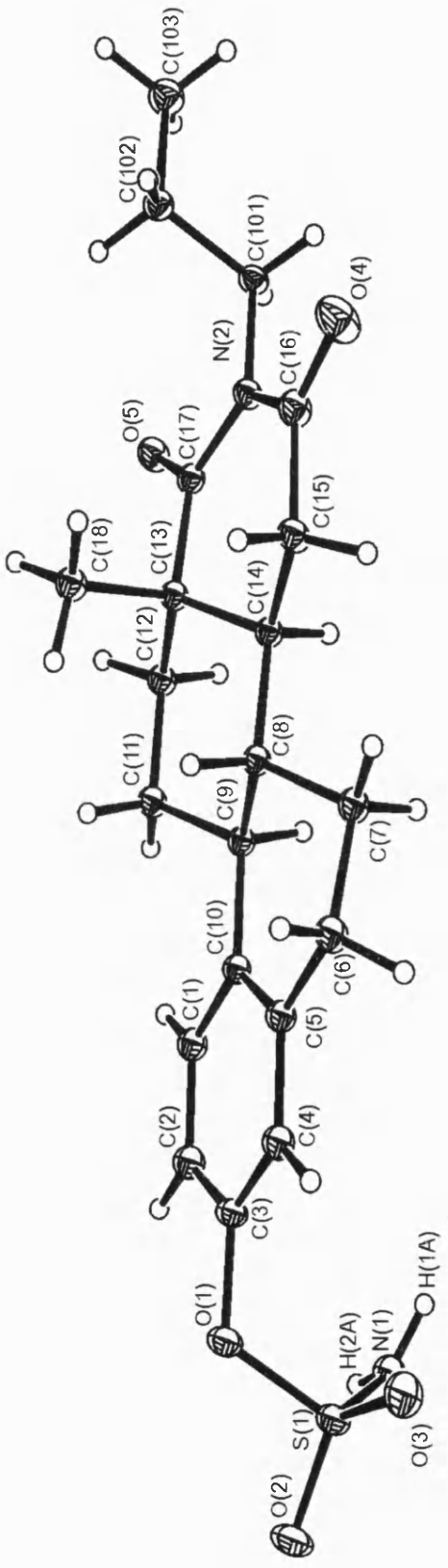
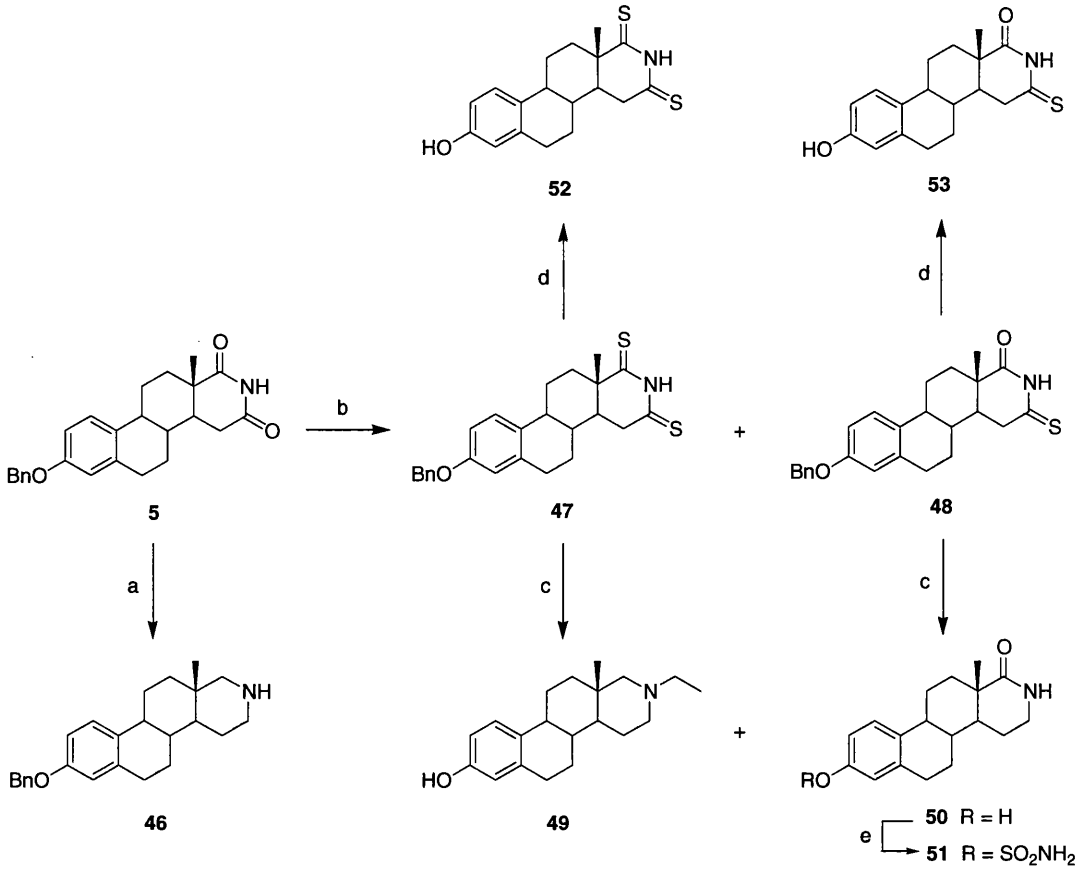


Figure 2.6 ORTEX[173] plot of the X-ray crystal structure of **35**. Ellipsoids are shown at the 30% probability level.

2.2.4 Further modifications on the D-ring

With the aim to expand the SAR on D-ring modified compounds and probe the pharmacological importance of several molecular features, we carried out further modifications on the 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide template. It was initially decided that the imido function would be reduced (totally or partially) in order to assess the role of the carbonyl groups and their effect on inhibitory activity.

In their study on steroidal D-ring lactams and imides, Matkovics *et al.* have reported a procedure for the reduction of **17** (cf. Table 2.1 p57) using $LiAlH_4$ in refluxing dioxane, in a Soxhlet extraction apparatus.[156] We decided to investigate this reaction on the benzylated precursor **5**, a more soluble substrate than the unprotected derivative **17** and therefore not requiring the use of a Soxhlet apparatus. Starting from compound **5** was also advantageous in that the reduced product can be selectively *N*-alkylated if desired. An attempt carried out in THF at room temperature only yielded a complex mixture of compounds from which no product could be isolated. Under refluxing conditions, the desired piperidine derivative **46** was obtained in disappointingly low 7% yield with separation from other reaction products proving problematic (Scheme 2.6). Its structure was assigned on the basis of ^1H NMR and mass spectrometry evidences, and confirmed by IR spectroscopy where the two anticipated characteristic imide carbonyl absorption bands of **5** at ν 1700 and 1720 were not seen. Switching solvent to dioxane, as reported in the original procedure, did not lead to any improvement in yield and therefore other reducing agents were evaluated for this reaction. Under milder conditions we hoped that a partially reduced derivative of **5**, where one or both carbonyls would have been converted to the corresponding alcohols would be available. Unfortunately, reactions of **5** with either BH_3.THF in THF or $NaBH_4$ in MeOH were unsuccessful. Alternatively, the stronger reducing agent 'super hydride' ($LiBEt_3H$ in THF) was employed, but still afforded no isolable product.



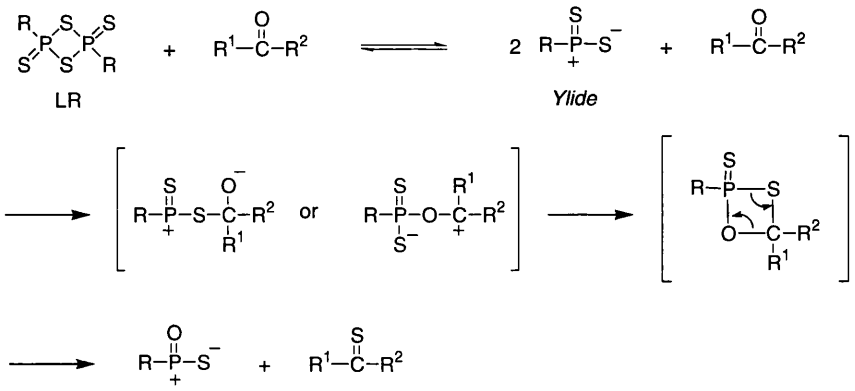
Scheme 2.6 Synthetic pathway to reduced derivatives of **17**. (a) $LiAlH_4$/THF, reflux; (b) Lawesson's reagent/toluene, reflux; (c) Ra-Ni/H_2O, EtOH; (d) BBr_3/DCM; (e) NaH/DMF, $ClSO_2NH_2$.

Because of the apparent difficulty in reducing the imide function of **5**, another pathway was envisaged to access the desired product(s). Carbonyl compounds can be reduced to alkanes via their corresponding thione under the action of Raney nickel, in particular this reaction can be applied to thioamides for the synthesis of amines.[174] It was therefore decided to convert the imide function of **5** into a dithioimide and then subject it to hydrogenolysis to effect simultaneous desulfurisation of the D-ring and debenzylation.

Lawesson's reagent (LR, Scheme 2.7) was used to thionate the imide carbonyl groups of **5**. This arylthionophosphine sulfide reagent is known to be an effective thiation agent, and affords high yield synthesis of thioketones and thioamides from the corresponding carbonyl compounds.[175,176] Recently, cyclic dithioimides were prepared using LR in boiling toluene.[177] Although the thiation of such imides was

reported to proceed rapidly using an excess of LR, it was noted that the thiation of sterically hindered carbonyls was slower.

The mechanism of the reaction is similar to that of the Wittig reaction, involving a 4-membered cyclic intermediate (Scheme 2.7).[176] Mechanistic studies have suggested that a highly reactive dithiophosphine ylide, rather than LR itself, is the active thiation agent. The cyclic intermediate may be accessed via two possible mechanisms: addition of the sulfur atom to the carbonyl group or attack of the carbonyl oxygen on the electrophilic phosphorus atom. The latter mechanism seems the most likely since it has been supported by structural, kinetic and spectroscopic studies.[178]



Scheme 2.7 Postulated mechanism for thiation of carbonyls using Lawesson's reagent, LR (R = ArOMe). The ylide may be the active thiation agent.[176]

When imide **5** was subjected to reaction with an excess of LR in refluxing toluene, two products of significantly different polarities were obtained. They were easily separated by flash chromatography and analysis of the two fractions by mass spectrometry indicated that the less polar compound was the expected dithioimide **47**, while the most polar fraction was that of a monothionated product. This was supported by ^{13}C NMR spectra analysis, where two highly deshielded C=S peaks could be seen at δ 202.03 and δ 214.03 for **47**, while peaks at δ 207.57 and δ 175.75 were observed for the other monothionated reaction product. Intuitively, this compound was assigned the structure **48**, where the less sterically hindered C=O has reacted with LR. An HMBC experiment, where 2-3 bond heteronuclear (^1H and ^{13}C)

correlations can be seen, confirmed this hypothesis since a strong correlation was observed between C18-H_3 and the C=O at δ 175.75 (Figure 2.7).

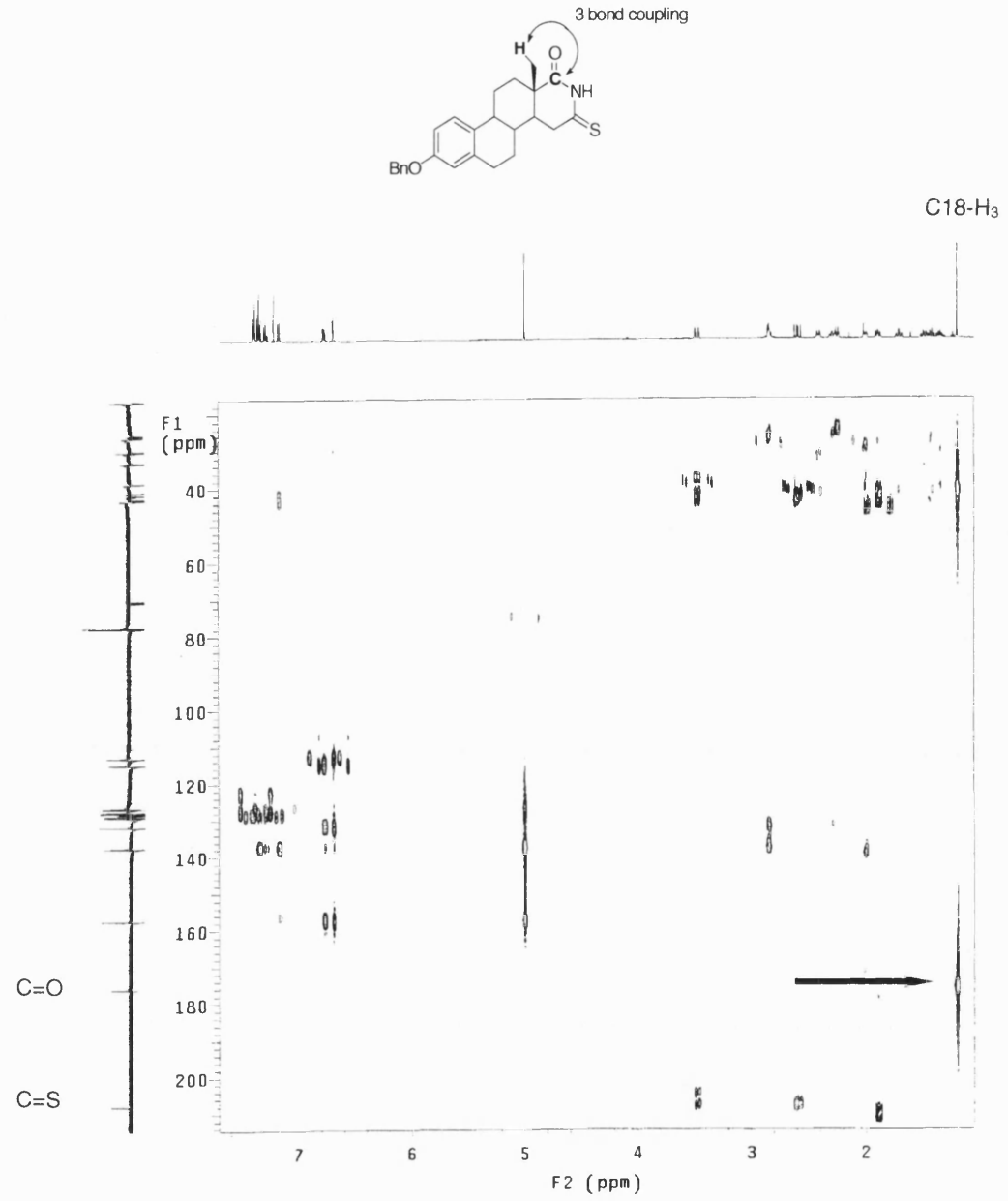
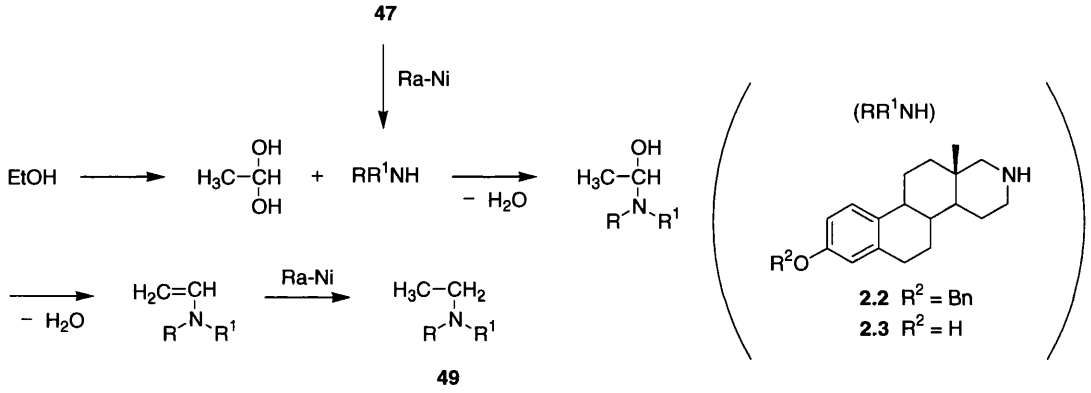


Figure 2.7 HMBC spectra of **48** ($CDCl_3$, 400 MHz). The arrow shows the cross peak between C=O and C18-H_3.

Both **47** and **48** were subjected to reaction with Raney nickel, in an aqueous ethanolic solution at room temperature overnight. While desulfurisation and deprotection of **48** afforded the D-ring lactam **50** in 73% yield, reaction of **47** under

the same conditions unexpectedly led to the formation of the *N*-ethyl piperidine derivative **49** in a low 24% yield. The presence of a triplet at δ 0.99 for a CH_3 group in the ^1H NMR spectrum of **49** as well as a characteristic 28 mass unit difference between the expected (NH) and the isolated (*N*-ethyl) product led to its structural identification. Examination of the literature provided an example of such side-reaction occurring between an amine and the solvent EtOH in the presence of Raney nickel.[179] An acetaldehyde resulting from oxidation of the solvent was postulated to react with an amine to yield an imine, which is then reduced *in situ* to a *N*-ethyl-amine.[179] In the reduction of **47**, a secondary amine is formed (RR^1NH, Scheme 2.8), affording an enamine upon reaction with the acetaldehyde. The latter can then be reduced to yield **49**. Changing solvent might therefore prevent the formation of **49** to give the desired product **2.3** or its benzylated precursor **2.2** (Figure 2.8)



Scheme 2.8 Side-reaction occurring in the Raney nickel (Ra-Ni) reduction of **47** in EtOH.

The lactam derivative **50** was then sulfamoylated, affording **51** in 31% yield. The synthetic pathway developed here to access **50** represents a shorter and more convenient synthesis of 6-membered D-ring lactam derivatives of E1 than the route proposed by Back *et al.*[157] In a synthetic study on novel 16-azaestrones and 17-aza-D-homoestrones, the authors reported the synthesis of the 3-methoxy derivative of **50** within 7 steps. Although the yields were in excess of 88% for each step, the forcing reaction conditions and the length of the synthesis made it less favourable for highly functionalised E1 derivatives.

The precursors **47** and **48** were also debenzylated using boron tribromide in DCM to afford **52** and **53** respectively in 50% and 33% yield. These mono and dithioimide

derivatives should serve as precursors for the synthesis of sulfur isosteres of the sulfamate **32** (cf. Table 2.1 p57). The activity of such compounds, as well as that of **51** should provide useful information on the role and importance of the carbonyl functionalities in the D-ring of these modified EMATE derivatives.

2.2.5 A-ring modification

Although the 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide template proved synthetically accessible for the rapid generation of target compounds, it was not known whether the resulting D-ring modified EMATE derivatives would be estrogenic. In a limited study on 3-hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivatives, Gupta and Jindal reported that the D-ring modification did not suppress *in vitro* estrogenic activity.[160] A-ring modification of EMATE proved to be a successful strategy for the production of non-estrogenic STS inhibitors, as exemplified by 2-MeOEMATE (Figure 2.8) which was devoid of estrogenic activity *in vivo*.[93,94] This compound was only 8 times less active than EMATE *in vitro*[93] and we were thus drawn to investigate the synthesis of 2-methoxy derivatives of our D-ring modified estrogens (Figure 2.8). In a preliminary study, it was anticipated that one example (R = H) would be synthesised and its biological activity evaluated.

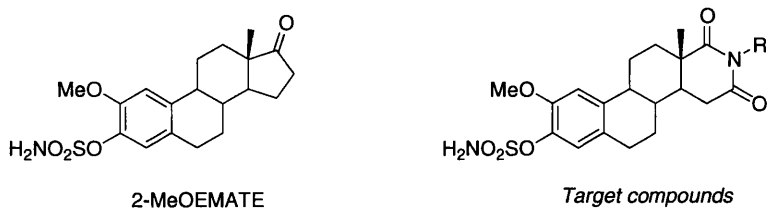
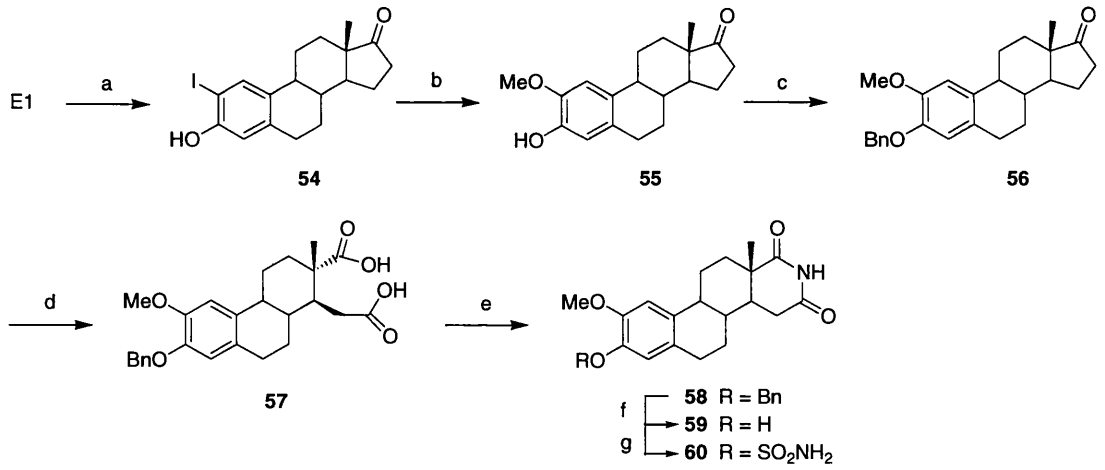


Figure 2.8 Structure of 2-MeOEMATE and A-ring modified target compounds (R = H or side-chain).

2-Methoxy estrogens are natural metabolites which have very low affinity for the ERs.[180] Considerable interest has emerged in recent years due to the wide range of biological activities exhibited by these compounds. For instance, 2-methoxy derivatives of E2 or analogues were found to display not only cytotoxic activity in cancer cells cultures,[92] but also antiangiogenic activity *in vivo*.[181] 2-MeOEMATE displayed similar biological properties, albeit at lower concentration. It induced G_2-

M cell cycle arrest, was an antimicrotubule agent *in vitro*[95] and was found to induce breast tumour regression *in vivo* in rats.[94]

The first synthetic approach to 2-methoxyestrogens was reported by Fishman,[182,183] wherein the final products were accessed with very low overall yield and an excessive number of steps. Most subsequent syntheses involved the direct conversion of 2-halogenated (2-iodo or 2-bromo) estrogens into the 2-methoxylated species. Mercury, thallium[184] and copper acetate[185] catalysed iodinations of E2 were investigated. Although several groups reported having achieved selectivity for halogenation and obtained 2-halo products in high yields, reproducibility of some results (selectivity and/or yield) was found to be problematic.[186] The conversion of 2-iodo estrogens into the corresponding 2-methoxy derivatives was studied by Numazawa *et al.*[187] wherein the reaction was reported to proceed in excellent yields when sodium methoxide was used in MeOH/pyridine, with copper chloride ($CuCl_2$) as a catalyst.



Scheme 2.9 Synthesis of 2-methoxy derivatives of D-ring modified estrogens. (a) $Hg(OAc)_2$, I_2, AcOH, 55°C to rt; (b) NaOMe/MeOH, $CuCl_2$, pyridine, reflux; (c) NaH/DMF, BnBr; (d) I_2, KOH, MeOH then KOH reflux; (e) urea, 180°C; (f) Pd/C, H_2, MeOH/THF; (g) NaH/DMF, $ClSO_2NH_2$.

In our group, 2-iodoestrone was recently obtained regioselectively from E1 using mercury acetate[188] as a catalyst (unpublished results). The regioselective halogenation was carried out in acetic acid at room temperature and was complete within 2 hours (Scheme 2.9). The ^1H NMR spectrum of the crude product indicated

the presence of the 4-iodo isomer and the starting material in minor proportions, and both were removed after two recrystallisations (in AcOH and EtOH), affording **54** in a 56% yield. The latter was reacted with a large excess of freshly prepared sodium methoxide in MeOH, in the presence of $CuCl_2$ in refluxing pyridine. 2-Methoxyestrone **55** was obtained with a yield of 78% and an overall yield of 44% from E1. This method is therefore highly effective, affording 2-methoxyestrone in two steps from E1, with a reasonable overall yield.

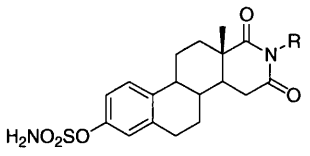
After benzylation of **55**, the resulting compound **56** was subjected to the haloform reaction, (cf. Scheme 2.2 p54). The limited solubility of the substrate in MeOH combined with difficult purification of the crude material led to a disappointing yield of 18% for **57**. Ring closure in presence of urea gave the imide derivative **58** in a 59% yield which, after subsequent debenzylation and sulfamoylation, was converted to the desired sulfamate **60**. A lack of reactivity was observed when the sulfamoylation was carried out in DMA, following Okada's procedure.[171] When conducted in DMF, in presence of NaH and a large excess of sulfamoyl chloride, the product **60** was isolated with a yield of 79%. This result could be due to steric hindrance at C3, resulting from the presence of the methoxy group at C2, although such lack of reactivity towards sulfamoylation in DMA is not seen with other 2-methoxy derivatives prepared in our group.

2.3 Results

2.3.1 *In vitro* inhibition of STS

The ability of the sulfamates synthesised to inhibit STS activity *in vitro* was examined in placental microsomes and the IC_{50} value for each compound is presented in Table 2.2. EMATE was used as a reference in this assay.

Table 2.2 IC_{50} values for inhibition of human placental steroid sulfatase by various D-ring modified steroid sulfamates and EMATE. Results are expressed as means of at least two determinations.



R	Compound	IC_{50} (nM)	R	Compound	IC_{50} (nM)
H	**32**	20	$(CH_2)_4Br$	**39**	12
CH_3	**33**	12	CH_2cyclopropyl	**41**	74
CH_2CH_3	**34**	52	CH_2Ar^tBu	**42**	23
$(CH_2)_2CH_3$	**35**	1	CH_2pyrid-3-yl	**43**	1
$(CH_2)_3CH_3$	**36**	382	CH_2Ar	**44**	3
$(CH_2)_4CH_3$	**37**	150	$CH_2CH{=}CH_2$	**45**	75
$(CH_2)_5CH_3$	**38**	288			
EMATE		18			

Of the different compounds tested, the steroids bearing a propyl **35**, a 1-pyridin-3-ylmethyl **43** and a benzyl **44** side-chain on the D-ring were the most potent, with IC_{50}s ranging from 1 to 3 nM. These compounds were found to be more potent than EMATE in the assay, with **35** and **43** being 18 times more active. In general, most of the analogues showed potent inhibition of STS activity.

Compound **32**, which has no side-chain at the *N*-atom, as well as **33** and **34**, the *N*-methyl and *N*-ethyl derivatives were found to be of similar potency to EMATE, with IC_{50}s between 12 and 52 nM. Unexpectedly, compounds **36**, **37** and **38** whose side-chains are respectively a *n*-butyl, *n*-pentyl and *n*-hexyl moiety, showed a dramatic decrease in potency (>10 fold) with IC_{50} values above 150 nM. This rather steep loss of potency suggests that the optimum hydrophobic interactions have been exceeded and steric hindrance is playing a role. Overall, good potencies were observed for compounds bearing non-linear side-chains and *tert*-butyl-benzyl **42**, 1-pyridin-3-ylmethyl **43**, and benzyl **44** derivatives had IC_{50}s below 25 nM. This indicates that restricted hydrophobic groups with less degrees of freedom are tolerated in the active site.

2.3.2 A-ring modification

Since 2-methoxy estrogens are less estrogenic than their parent, and in particular, 3-sulfamoylated derivatives were shown to additionally affect cell growth *in vitro*,[95] both **59** and **60** were assessed for their antiproliferative activity on MCF-7 breast cancer cells (Table 2.3).

Table 2.3 IC_{50} values for inhibition of MCF-7 cell growth by **59** and **60**, and inhibition of STS in placental microsomes by **60**. Results are expressed as means of at least two determinations (± S.D. for inhibition of MCF-7 cell growth).

Compound	IC_{50} μM (inhibition of cell growth)	IC_{50} nM (inhibition of STS)
59	42.19 ± 17.43	nd
60	24.28 ± 14.50	309
667 COUMATE	Nd	3
2-MeOEMATE	0.15 ± 0.05	30[a]

[a]from Purohit *et al.*[93]

Unfortunately, **59** and **60** showed poor growth inhibitory properties, with IC_{50} values of 42 and 24 μM respectively, making them at least 160 fold less active than 2-MeOEMATE. When examined in placental microsomes for activity against STS, **60** had an IC_{50} of 309 nM and 667 COUMATE, with an IC_{50} of 3 nM, was 100 times more active.

2.3.3 *In vitro* estrogenicity

In order to determine whether the compounds developed could be potentially used for the treatment of estrogen-sensitive breast cancer, their estrogenic potency was initially assessed *in vitro*. Given that hydrolysis of the sulfamate moiety might occur *in vivo* by *(a)* chemical hydrolysis or metabolism or *(b)* after irreversible inhibition of STS by the sulfamate moiety,[88,90,91,118] both the sulfamates and their corresponding phenolic derivatives were assessed for estrogenicity.

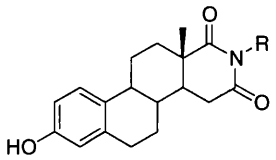
A luciferase reporter gene assay was used to determine the ability of each compound to bind to the ER. The compounds were screened at four concentrations: 100 pM, 1 nM, 10 nM and 100 nM alongside E2 concentrations of 1 pM, 10 pM, 100 pM and 1 nM. The results, summarised in Table 2.4, give the lowest concentration at which estrogenic activity was detected for each compound i.e. the lowest concentration for which above background level of luciferase-induced luminescence was observed.

Table 2.4 Lowest concentration at which estrogenic activity was detected (lowest conc.) for the phenolic derivatives **17-28** and **31**, their corresponding sulfamates **32-39** and **41-45** and EMATE.

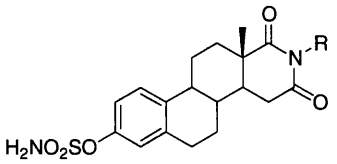
PHENOLS		SULFAMATES	
Compound	lowest conc. (nM)	Compound	lowest conc. (nM)
17	1	**32**	100
18	100	**33**	>100
19	100	**34**	100
20	>100	**35**	>100
21	>100	**36**	100
22	n.d.	**37**	>100
23	>100	**38**	>100
24	>100	**39**	>100
25	>100	**41**	>100
26	>100	**42**	100
27	>100	**43**	>100
28	nd	**44**	>100
31	10	**45**	>100
E2	0.01	EMATE	10

Most of the compounds tested displayed estrogenicity at concentrations of 100 nM or above and can therefore be considered as weak estrogens. They are 10^4-fold less estrogenic than E2, whose activity was detected at 10 pM. The most estrogenic compounds were the phenolic derivatives **17** and **31**, with activity detected at 1 and 10 nM respectively. In this assay, their estrogenic potency was comparable to that of EMATE, which is highly estrogenic *in vivo*.[90,91]

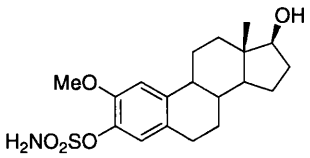
The assay was repeated for the compounds which had a detectable activity at a concentration of 100 nM or lower, namely the phenolic derivatives **17**, **18**, **19** and **31** the sulfamates **32**, **34**, **36** and **42** (Figure 2.9). The compounds were tested at three concentrations: either 1 nM, 10 nM and 100 nM or 10 nM, 100 nM and 1 μM. The results are expressed as a percentage of the activity of E2 at 10 pM for each concentration tested (Figure 2.10). 2-Methoxy-estradiol-3-*O*-sulfamate (2-MeOE2MATE, Figure 2.9), which recently showed to be non estrogenic *in vivo*,[84] was used as a reference in this assay.



17 R = H
18 R = CH_3
19 R = CH_2CH_3
31 R = $CH_2CH{=}CH_2$



32 R = H
34 R = CH_2CH_3
36 R = $(CH_2)_3CH_3$
42 R = CH_2Ar^tBu



2-MeOE2MATE

Figure 2.9 Structure of the compounds tested for their estrogenic activity at three concentrations.

Clearly, only one compound (**17**) displayed high estrogenic activity *in vitro*. At a dose as low as 1 nM, compound **17** was already as potent as 10 pM of E2. Its sulfamate **32** was also a potent estrogen, although to a lesser extent, since its estrogenicity was comparable to that of 10 pM of E2 at a concentration of 10 nM. The other compounds tested were all less active than 2-MeOE2MATE, which was estrogenic at 10 nM. Compounds **18**, **19**, **34**, **36** and **42** are therefore at least 10^3-fold less estrogenic than 10 pM of E2.

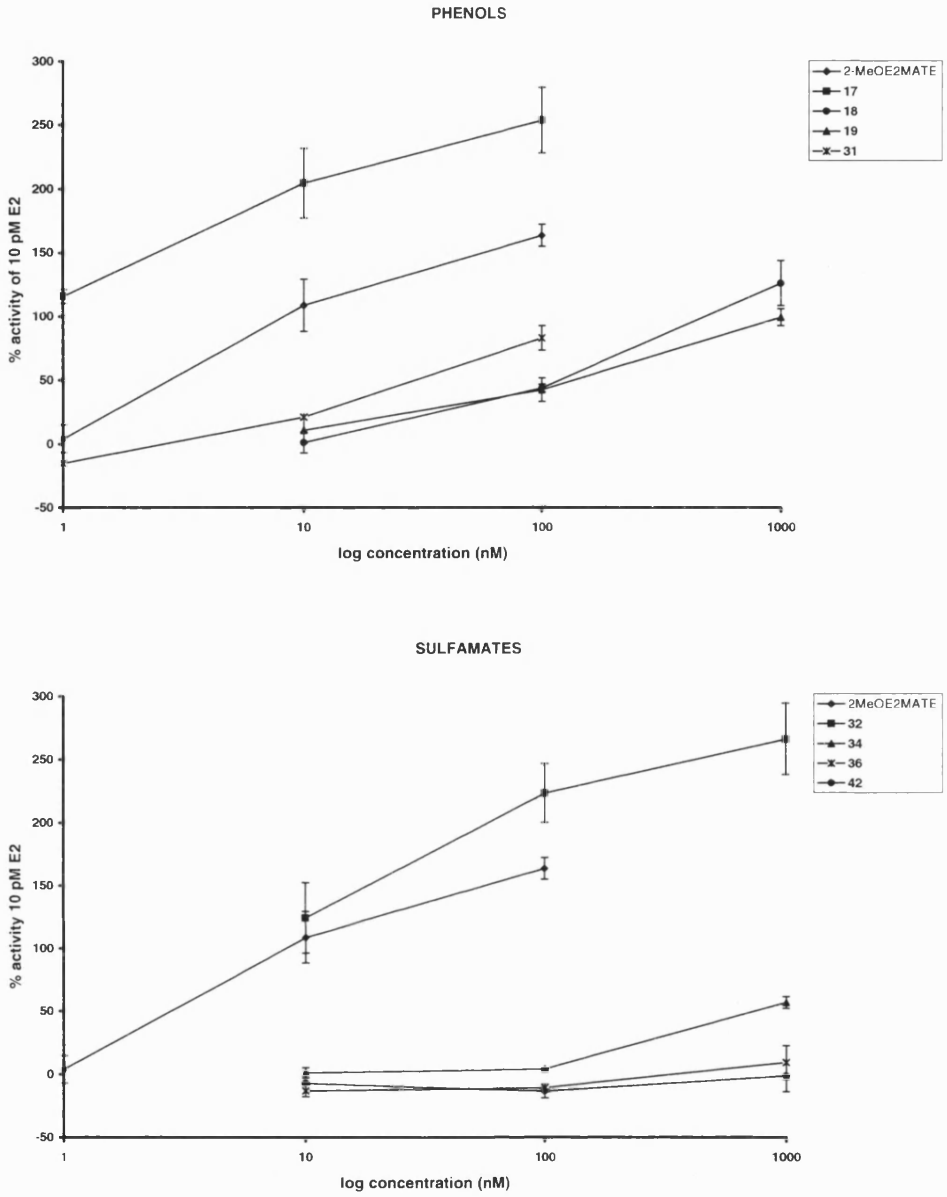


Figure 2.10 Estrogenicity of **17-19**, **31**, **32**, **34**, **36** and **42** expressed as a percentage of the activity of E2 at 10 pM. Results are expressed as means ± S.D. triplicate measurements.

The ability of some compounds to inhibit the effect of 100 pM of E2 was also examined in order to assess their anti-estrogenic potential in the luciferase assay (Figure 2.10). Eight compounds, including the two most potent STS inhibitors **35** and **43**, were tested at three different concentrations (1 nM, 10 nM, 100 nM). Raloxifene (Ral), a known antiestrogen was used as a reference in this assay.

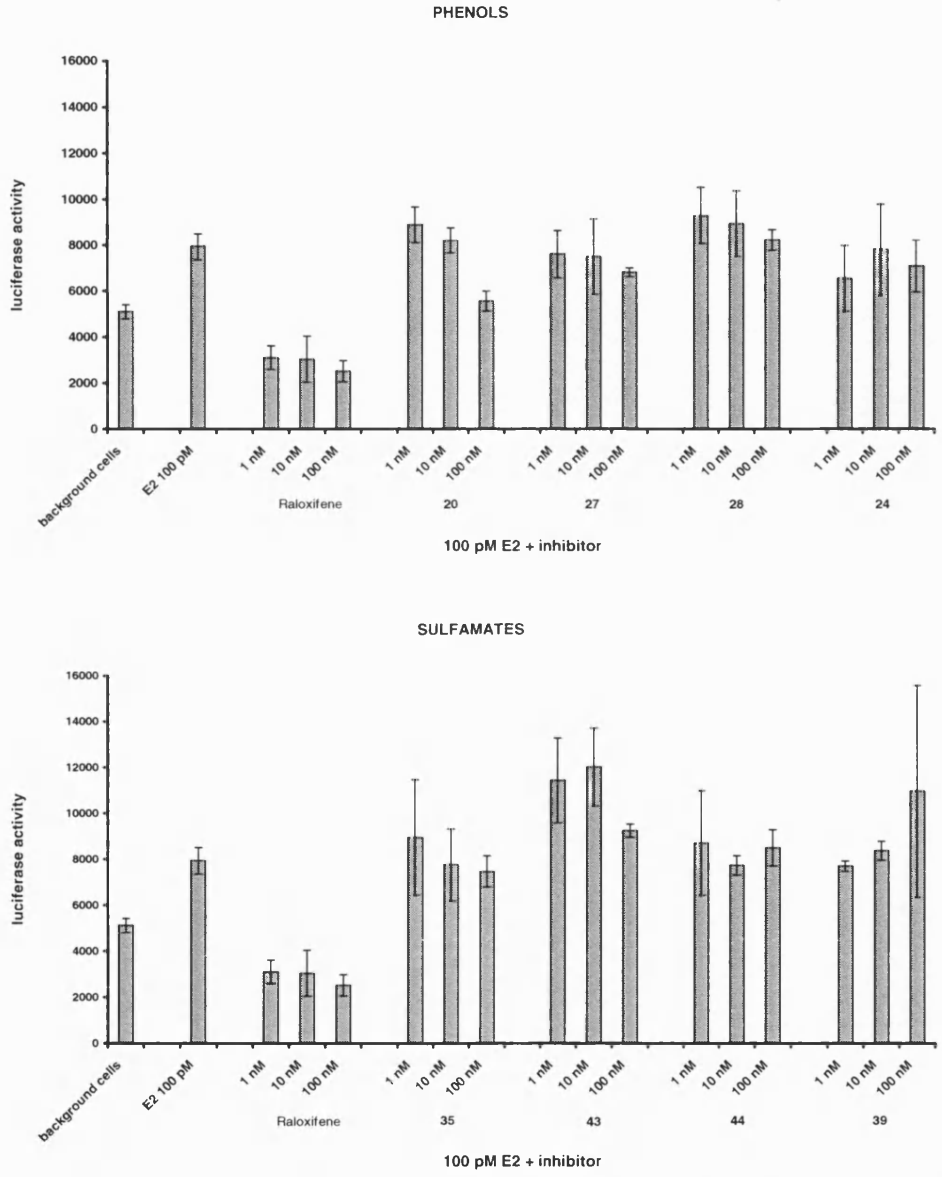


Figure 2.10 Inhibition of the estrogenic effect of 100 pM of E2 by the phenols **20**, **27**, **28**, **24** and their sulfamates **35**, **43**, **44**, **39** at 1, 10 and 100 nM. Raloxifene is used as a reference. Results are expressed as means ± S.D. triplicate measurements.

While Raloxifene could inhibit the activity of the 100 pM dose of E2, none of the tested compounds displayed clear antiestrogenic properties to the same degree. The only potential antiestrogen is **20**, where a reduction in estrogenic activity is observed at 100 nM. The luciferase activity drops from a level comparable to that of E2 (at 100 pM) to a near background level upon application of 100 nM of **20**.

2.3.4 *In vivo* studies

In vitro evaluation of the D-ring modified compounds identified two highly potent STS inhibitors. With IC_{50} values of 1 nM, **35** and **43** were 18-times more active than EMATE in placental microsomes. These compounds were selected for *in vivo* studies in order to assess their inhibitory activity on rat liver STS and examine their ability to stimulate uterine growth in the ovariectomised animals as a measure of their estrogenic potential.

After treatment of ovariectomised rats with an oral dose of 10 mg/kg/day of either inhibitor for 5 days, an almost complete inhibition of liver STS activity (99%) could be observed (Figure 2.11A). EMATE, which was tested at a 200-fold lower dose (50 µg/kg/day) subcutaneously gave a 73% inhibition of STS activity in the liver.

Both **35** and **43** were not estrogenic *in vivo*. Unlike EMATE, they had no effect on uterine growth in ovariectomised rats and the ratio of the uterine weight/body weight was the same as in the animals receiving vehicle only (Figure 2.11B). The much lower dose at which EMATE was tested was sufficient to produce a marked increase in uterine weight (>200%) in these animals, attesting the high estrogenic potential of this molecule.

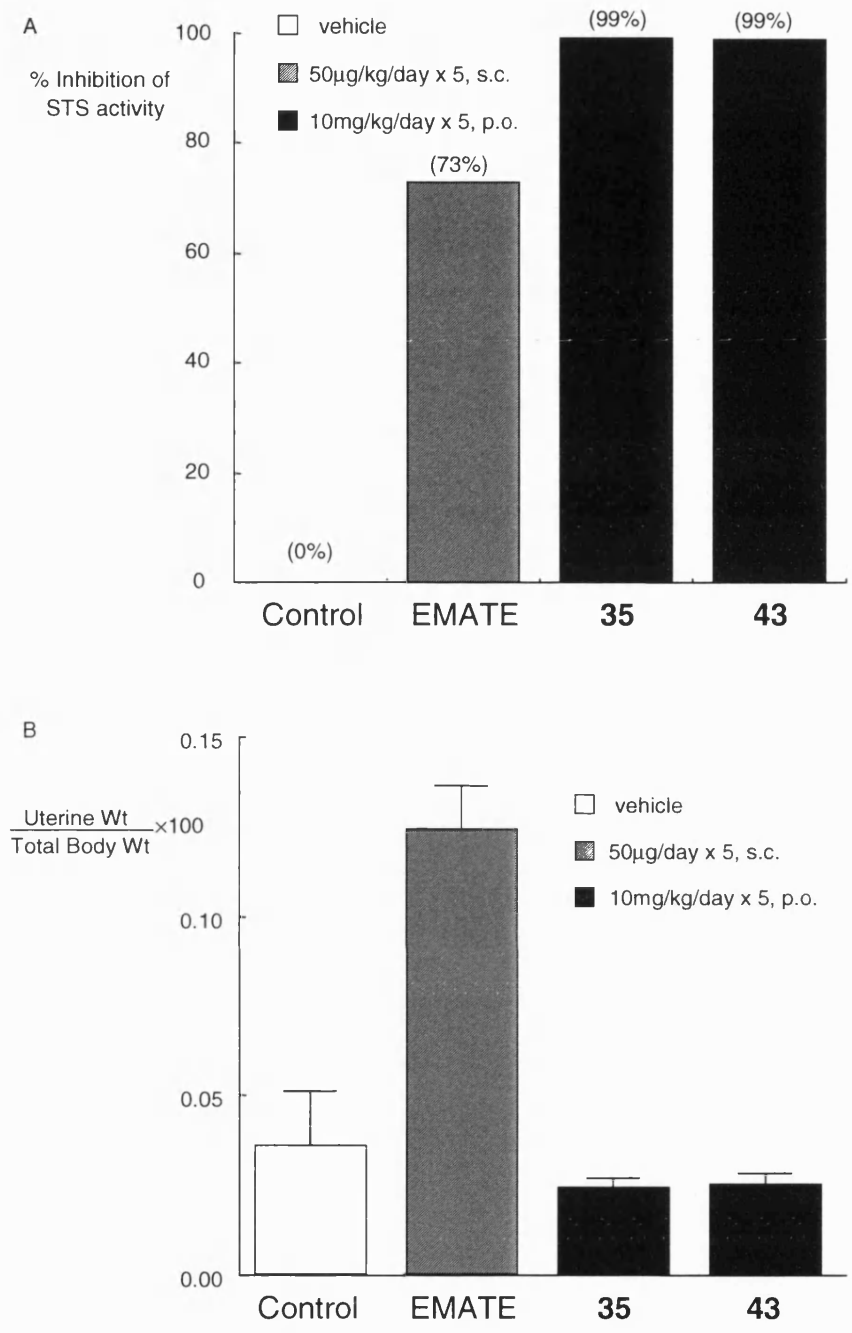


Figure 2.11 A, Inhibition of rat liver STS activity by **35** and **43**, means ± S.D. (for **35** and **43** the S.D.s were <10%). Basal sulfatase activity in liver homogenates of untreated animals was 8.75 nmol/h/mg protein. Figures in parentheses represent the percent inhibition compared with the control. B, Effect of **35** and **43** on uterine growth in the ovariectomised rat. Results are expressed as mean ± S.D. of triplicate measurements.

2.4 Discussion

2.4.1 STS inhibition *in vitro*

The most advanced steroid sulfatase inhibitor to date, 667 COUMATE, has just entered phase I clinical trials and has the potential to be the first in class to reach the market. So far, no steroidal inhibitor has achieved the same degree of inhibition *in vivo*, combined with non estrogenic properties and oral availability. Efforts have therefore been concentrated on developing potent, non estrogenic, steroidal inhibitors of STS, as back-up clinical candidates to 667 COUMATE with a similar or improved drug profile.

A report in the literature that D-ring modifications to EMATE could reduce its estrogenicity, without loss of potency prompted us to investigate further such D-ring derivatives.[98] The established pharmacophore for STS inhibition, an aryl-3-*O*-sulfamate moiety, was retained and a template was chosen so that rapid investigation of the SAR around the D-ring would be achieved. Several published SAR on STS inhibitors have stressed on the importance of the hydrophobicity of the side-chains introduced on the D-ring for inhibition.[99,100,102] It is believed that such moieties enhance the activity of the compounds by increasing their affinity for the enzyme and the active site, which presumably contains a hydrophobic pocket. A number of D-ring modified analogues were prepared from the 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide template chosen for our inhibitors and the activity of the corresponding sulfamates was examined in placental microsomes.

A series of linear alkyl side-chains of increasing length (C_1 to C_6) was first introduced on the D-ring, resulting in compounds **32-38**. A plot of their IC_{50}s vs. the number of CH_2 units in the side-chain is depicted in Figure 2.12. Derivatives bearing short side-chains (**33-35**) were found to be potent inhibitors of STS, with IC_{50}s in the range of that of EMATE. In particular, the *n*-propyl analogue **35** was found to be highly potent with an IC_{50} of 1 nM, being 18-times more active than EMATE in the *in vitro* assay. In contrast, a sharp decrease in potency was observed for **36-38**, with

IC_{50}s above 150 nM. A 400-fold drop in activity was observed when the number of carbon atoms in the side-chain increased from C_3 to C_4. We were pleased to see that the non alkylated derivative **32** was equipotent to EMATE, suggesting that the replacement of the D-ring of EMATE with a six-membered piperidinedione ring has not been detrimental to its inhibitory activity. No doubt a major factor for the potency of this series of compounds is the aryl-3-*O*-sulfamate moiety, but activities higher than that of EMATE for **33** or **35** indicate that the D-ring substituents might contribute to the inhibition by enhancing the affinity for the enzyme.

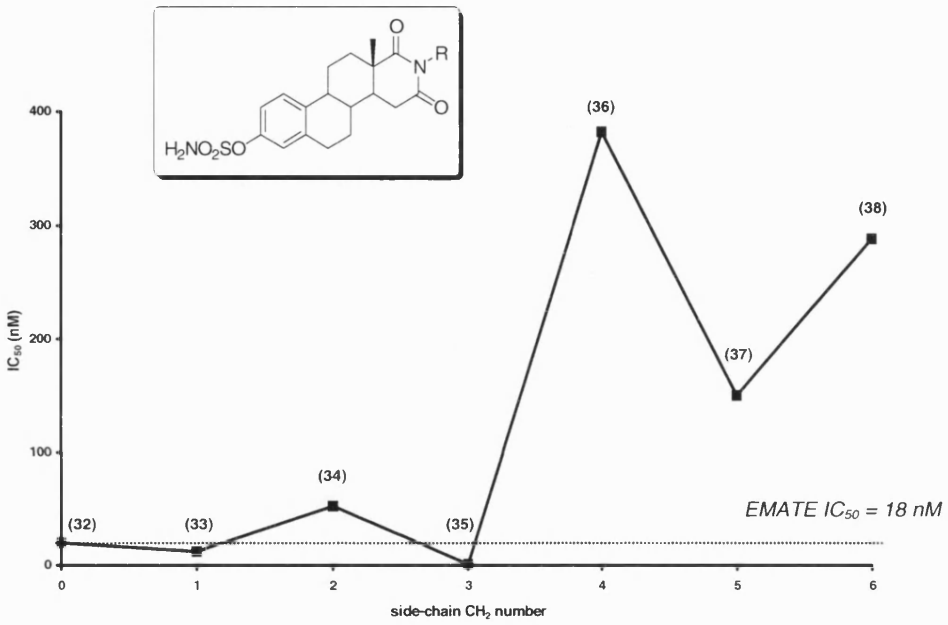


Figure 2.12 Effect of various side-chain lengths on the inhibitory potency of 3-sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide derivatives **32-38** (R = H or linear alkyl side-chain; compounds numbers are in parentheses).

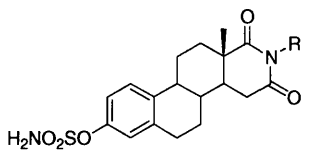
Two factors might explain the decrease in potency observed for *n*-butyl compounds onwards: steric bulk and hydrophobicity. Higher conformational flexibility of longer alkyl groups might have prevented adequate fitting of inhibitors such as **36-38** into the active site. It is also possible that the hydrophobic pocket which these alkyl substituents are probably exploiting has in fact a limited capacity where only short side-chains (up to C_3) can fit. If steric clashes occur between the alkyl side-chain and a part of the enzyme active site, this results in destabilisation of the enzyme-inhibitor complex and therefore lowers the affinity, hence the activity of the inhibitor.

Most likely, the nature of the interaction of the side-chains of **32-38** with the active site is hydrophobic. In a recent study on a series of 4-sulfamoylated phenyl ketones, Ahmed *et al.* confirmed the importance of hydrophobicity for potent STS inhibition. The authors also showed a strong correlation between the IC_{50}s for STS inhibition (in placental microsomes) and logP values of the phenyl ketones inhibitors.[107] The presence of an optimum logP value for these analogues is indicative of an alkyl chain length limit beyond which the potency of the inhibitors decreases. If logP is too high, entry of the sulfamates into the STS active site is disfavoured, which consequently lowers the inhibitory activity. On the other hand, a too hydrophilic character diminishes the ability of the compounds to partition into a non-polar environment.

For the derivatives **36-38**, it is therefore possible that too high logP values are reached for these inhibitors to enter the active site. It is also conceivable that the hydrophobicity of the substituents increases the inhibitory activity of the D-ring imide derivatives, but the steric factors contribute to the opposite effects for those more bulky side-chains. In particular steric clashes might be too important for linear substituents where the number of carbon atoms exceeds 3. Clearly, the best compromise between hydrophobicity and steric bulk is obtained with the propyl side-chain (**35**, Figure 2.12).

Several other SAR have been reported where similar patterns were observed. On the development of non steroidal inhibitors, Woo *et al.* synthesised a series of tricyclic coumarin sulfamates in order to optimise the size of the third ring.[104] The best inhibitors had a 7- and a 10-membered ring, and a marked reduction in activity was observed for larger ring sizes. Derivatives of E2 bearing linear side-chains at C17α reached a maximum of activity for the octyl moiety.[99,102] Li's 17β-alkanoyl and alkycarbamoyl derivatives of EMATE had a side-chain length limit and the best inhibitors had a total of 8 carbon atoms.[98]

Table 2.5 IC_{50} values for inhibition of placental STS by the D-ring modified compound **39**, **41-45**.



R	Compound	IC_{50} (nM)	R	Compound	IC_{50} (nM)
$(CH_2)_4Br$	**39**	12	CH_2pyrid-3-yl	**43**	1
CH_2cyclopropyl	**41**	74	CH_2Ar	**44**	3
CH_2Ar^tBu	**42**	23	$CH_2CH{=}CH_2$	**45**	75

In light of the encouraging results obtained for the linear alkyl side-chain derivatives, we decided to introduce different moieties while retaining the overall hydrophobic character of the side-chains. To examine the effects of increasing hydrophobicity without increasing the length, we synthesised the *N*-bromobutyl analogue **39**. With a side-chain length similar to that of **36**, its activity was more than 30 times higher, indicating the importance of hydrophobic interactions in this region of the active site. The *N*-allyl derivative **45** was also prepared. Although not as potent as **35**, the additional rigidity conferred by the allyl moiety was not too detrimental to the activity (IC_{50} = 75 nM). This was also exemplified by compound **41**, the cyclopropyl analogue, whose inhibitory activity was similar to that of **45**. We then undertook the synthesis of the *N*-benzyl derivatives **42** and **44**, relying on the fact that a benzyl group *(a)* contains an allylic portion (cf. **45**) *(b)* is more hydrophobic and less sterically restrictive with its total of seven carbons than would be a linear heptyl side-chain. Both **42** and **44** proved the validity of this approach as they were found to inhibit STS activity with IC_{50}s of 23 nM and 3 nM respectively. This further asserts that the hydrophobic pocket around the D-ring in the active site tolerates steric bulk in a certain conformation, as already demonstrated by Poirier *et al.* with their potent 17α-benzyl derivatives of E2.[99,102] Compound **43** was also prepared on the basis that the nitrogen atom of the pyridyl moiety would possibly enhance the affinity for the active site via hydrogen bonding. With an IC_{50} of 1 nM, **43** was as potent as **35**, proving that the effect of a linear propyl side-chain is equivalent to that of a pyridyl moiety in the vicinity of the STS active site. It is likely that the nitrogen-containing

part of the side-chain of **43** points away from the hydrophobic region, while the hydrophobicity is due to rest of the side-chain.

With **35** and **43** being 18 times more potent than EMATE and 8 times more potent than 667 COUMATE *in vitro*, our study resulted in two novel and potent lead compounds with side-chains which differ both chemically and sterically. Although there is no definitive proof of the nature of inhibition, it is anticipated that the sulfamate moiety leads to an irreversible type of inhibition of STS. Clearly, the modified D-ring and its substituents have a role in the inhibition and the overall result can be seen as a combination of the effects of the pharmacophores present at both ends of the molecule. Similarly, in the development of 17α-benzyl derivatives of EMATE, Ciobanu *et al.* observed an 'additive-like' inhibitory effect of the two kinds of substituents on the A- and D-ring.[100] The authors postulated that the substituents at C17α facilitate the rate of binding, leading to more potent inactivation of the enzyme. It was also suggested that one molecule of the inhibitor may act on two molecules of enzyme, because hydrolysis of the inactivating sulfamate group leads to the corresponding phenol that can then exert its inhibitory effect on neighbouring free enzymes, resulting in an overall better efficacy.[100] Whether this is the case or not for our inhibitors is not known at present, but it is conceived that once the irreversible type of inhibition is established, the phenol derivatives **17-28** and **31** could be tested for STS inhibition.

Finally, when the activity of **60** (the 2-methoxy analogue of **32**) was examined in placental microsomes, it was found to be a poor inhibitor of STS. With an IC_{50} of 309 nM, it was 100-fold less active than 667 COUMATE in the same assay and some 15 times less potent than its non-methoxylated analogue **32**. It is possible that the methoxy group causes a displacement of the sulfamate group in the active site resulting in a low affinity binding. The 2-methoxy moiety may also conceivably shield the sulfamate group, possibly via hydrogen bonding.

2.4.2 Estrogenicity and antiestrogenicity studies *in vitro*

Because of the intrinsic estrogenicity of EMATE, we introduced D-ring modifications on the estrone-3-*O*-sulfamate skeleton that were, to some extent, intended to reduce this problem. So far, it has not been elucidated whether the estrogenicity of EMATE is a direct effect of the sulfamate moiety or of E1 and its metabolites, although it is not expected that sulfamates directly bind to the ER. This was corroborated by the findings of Elger *et al.*, which indicated that estradiol-3-*O*-sulfamate, and possibly estrogen sulfamates in general, did not have affinity for the ER *in vitro*.[91] In a recent study on chromenone-sulfamate-based STS inhibitors, Nussbaumer *et al.* observed that EMATE stimulated the growth of MCF-7 cells but had a weak affinity for ERα and ERβ, whereas the corresponding phenol, E1 and its metabolite E2, showed high affinity for the ERs.[110] Since E1 may be formed from hydrolysis of EMATE *in vivo*, it is important to take into account the phenolic derivatives **17-18** and **31** as well as the sulfamates **32-39** and **41-45** in our study on estrogenicity.

The assay was carried out using the MCF-7 human breast cancer cell line, which contains ER and is estrogen responsive. By ER, we imply ERα although a second isoform, ERβ, has recently been discovered.[30] It is still unclear how each isoform modulates the estrogenic response and interacts with different compounds in various tissues, therefore our study was limited to ERα. In the series of phenols, the most estrogenic compound was the unsubstituted analogue **17**, with activity detectable at 1 nM (Table 2.4 p72). It was 10 times more estrogenic than EMATE in the same assay and therefore displayed high affinity for the estrogen receptor. The fact that the non-alkylated template was found much more estrogenic than the compounds bearing a side-chain corroborates the hypothesis that the introduction of a hydrophobic moiety on the D-ring can contribute to reducing the estrogenicity of the precursor. The compounds bearing small and non-bulky side-chains (**18**, **19** and **31**) had a higher estrogenic activity than others, with activity detectable at 10 nM for the *N*-allyl analogue and at 100 nM for the *N*-methyl and *N*-propyl derivatives. Again, this is in accordance with literature reports, where length and bulkiness are important factors when aiming at no estrogenic activity.[97,101,152] As expected, none of the sulfamates

were estrogenic and it is assumed that they do not bind to the ER. In particular, we were pleased to see that our two lead compounds, **35** and **43** did not display any estrogenic activities at concentrations as high as 100 nM.

When the compounds that displayed estrogenic activities at 100 nM and below were re-examined against the estrogenicity of 10 pM of E2, all the analogues except one, were less estrogenic than 2-MeOE2MATE, used as a reference in the assay. Given that 2MeOE2MATE is not estrogenic *in vivo*,[84] none of these compounds would be expected to bind strongly to the ER if used in living models. Clearly, the compound with no substitution on the D-ring was an agonist of the ER, be it in its phenolic **17** or sulfamoylated form **32**. In particular, at a concentration of 1 nM, **17** was already as estrogenic as 10 pM of E2. Interestingly, with **32** being estrogenic, we can assume that this D-ring modified estrogen sulfamate does bind to the ER, and its affinity for the ER is possibly driven by interactions of the receptor with the D-ring moiety. It is also conceivable that **32** is hydrolysed into **17** during the assay.

We were interested to see if some of the compounds that did not display estrogenic properties did bind to the ER, and thus behaved as antagonists. To this end, increasing doses of each inhibitor were assessed for their ability to inhibit the activity of 100 pM E2. For comparison, the antiestrogen Raloxifene was also tested in this assay. None of the compounds tested (the phenols **20**, **27**, **28**, **24** and the sulfamates **35**, **43**, **44**, **39**) were clearly antiestrogenic. While raloxifene could block the estrogenicity of 100 pM of E2, for most of the compounds, the luciferase activity recorded at various concentrations of inhibitor was similar or higher than that of 100 pM of E2. The only compound displaying signs of antagonism of the ER was the phenol derivative **20**. Unlike all the other compounds tested, this *N*-propyl analogue does bind to the ER and prevents the access of E2 at a concentration of 100 nM. From the previous test results, we also know that **20** is not estrogenic at such a concentration. Further testing would be required to assess the full potential of this derivative as an antiestrogen.

From the different sets of data, it appears difficult to rationalise the ability of our compounds to elicit an estrogenic response. Clearly, most of the STS inhibitors

synthesised are devoid of intrinsic estrogenic activity, which further asserts the validity of the D-ring modified template we chose. However, it is still unclear whether the apparent lack of estrogenicity of the compounds is the result of their lack of affinity for the ER, or the effect of a weak antagonistic behaviour. Examination of the recent literature could provide some insight into the mechanisms involved in modulation of the ER.

With the resolution of the three-dimensional structure of the ER-LBD bound with agonists and antagonists,[41,42] several groups have now proposed a structural basis for agonism and antagonism.[40,41] From the crystal structure, it appears that the binding of E2 and other agonists of ERα is mediated through a combination of specific hydrogen bonds and that a tight complementarity exists between the binding cavity and the steroid's non-polar surfaces. Both extremities of the steroid establish direct hydrogen bonds with critical residues of the LBD, the 3-OH with Glu353 and Arg394 and the 17-OH with His524. Mutagenesis studies have shown that residue Glu353 plays an important role in the binding of the A-ring phenolic group of E2,[189] thus explaining the higher affinity of phenolic derivatives for the ER as compared to that of sulfamates. Comparison of the crystal structure of the LBD of ERα in complex with E2 and raloxifene showed that both agonists and antagonists bind at the same site within the LBD, but the binding mode is different.[41] Whether ER ligands are agonists or antagonists depends on the position adopted by one of the α-helices of the protein (H12) which is important for hydrophobic interactions. Steroidal or non-steroidal antiestrogens that possess a long hydrophobic or bulky side-chain displace this helix, therefore disrupting the overall topography of the complex. It is therefore anticipated that compounds such as **17** can bind to the ER without disrupting H12 (i.e. in a similar mode to that of E2), as exemplified by its estrogenic activity. On the other hand, at concentrations up to 100 nM, compounds bearing long or bulky side-chains, such as the phenolic derivatives **27**, **28** or **24**, do not bind to the ER. It is assumed that the propyl side-chain of **20** is short enough to afford binding, yet long enough to disrupt H12 and thus results in an antagonist.

2.4.3 *In vivo* evaluation of 35 and 43

Being 18 times more active than EMATE in placental microsomes and devoid of estrogenic activity in preliminary *in vitro* testing, **35** and **43** were selected for *in vivo* studies. Oral administration of both inhibitors in ovariectomised rats at 10 mg/kg/day for a period of 5 days resulted in a complete inhibition (99%) of liver sulfatase activity. At a 200-fold lower dose, EMATE achieved a 73% inhibition of STS activity. These results are in agreement with the excellent *in vitro* potencies observed for the selected inhibitors. At the high dose of 10 mg/kg/day, sulfatase activity is completely inhibited by both compounds and further studies employing a range of lower doses will be required in order to fully evaluate the comparative potencies of **35** and **43**.

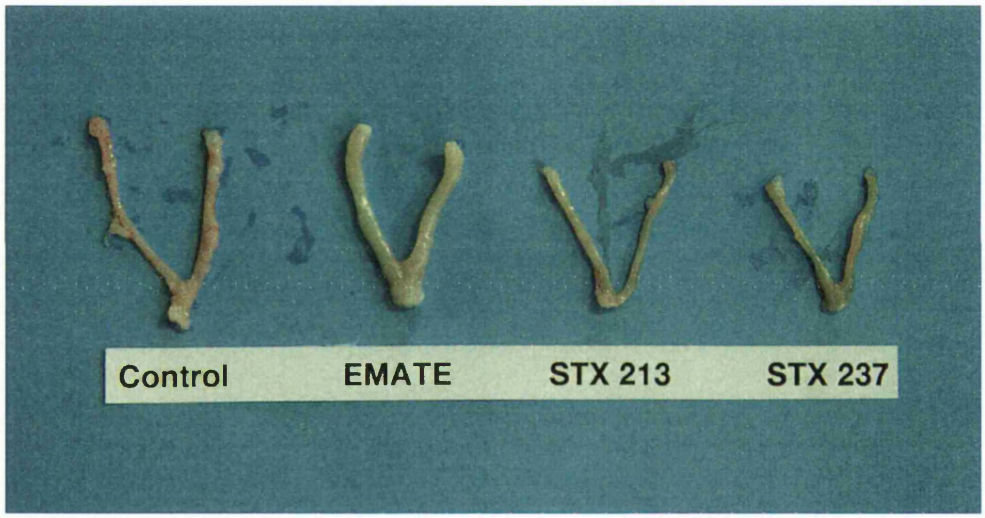


Figure 2.13 Uteri of ovariectomised rats treated with EMATE (50 µg/kg/day, s.c.), **35** (STX 213) and **43** (STX 237) (10 mg/kg/day, p.o.) for 5 days. Control animals received vehicle only.

In the same study, using an ovariectomised rat uterus weight gain assay, the *in vivo* estrogenicity of the compounds was studied. In animals receiving vehicle only, the average uterine weight ratio (expressed as a percentage of uterine weight/total body weight) was found to be 0.05. Upon treatment with EMATE for 5 days at 50 µg/kg/day (s.c.), a 200% increase in uterine weight could be observed (ratio > 0.10) in comparison with control group. Both **35** and **43** lacked estrogenicity when applied

orally at 10 mg/kg/day, resulting in uterine weight ratios similar to or lower than the controls. A picture of the uteri excised of fat gives a visual account of the effects induced by a highly estrogenic compound such as EMATE (Figure 2.13).

To be successfully developed as anti-cancer drugs, it is advantageous if compounds are active on oral administration. Although the *in vivo* results obtained for **35** and **43** tend to indicate that they are both orally active, we do not have any data attesting of their bioavailability. To ensure that such compounds reach the systemic circulation without undergoing significant degradation, metabolic studies would be required. Nevertheless, it is conceivable that such D-ring modified estrogens are orally available because they also are sulfamates. Natural estrogens have a poor oral bioavailability, whereas sulfamoylated derivatives of estrogens, such as EMATE, have enhanced oral availability.[90,91] In their investigations on estrogen sulfamates and their potential application in oral hormone therapy as prodrugs of their parent estrogens, Elger and coworkers found that sulfamoylation of E2 increased its activity by 100-fold on oral application. This property of sulfamates is thought to result from their ability to be taken up by red blood cells (rbcs) after absorption and transported through the liver without undergoing metabolism.[91] This also explains why EMATE is active at low doses orally, in contrast to other estrogens that undergo substantial metabolism and inactivation, necessitating the use of high doses to achieve biological effectiveness. Possibly, **35** and **41** might achieve a high level of inhibition of STS at lower doses and further testing will be required.

Recent reports in the literature suggest that the partitioning of EMATE into rbcs may be due to its reversible binding to carbonic anhydrase II (CA II), which is present in the cytosol of erythrocytes.[190,191] As several CA II inhibitors posses a sulfonamide group, it was reasoned that compounds such as EMATE or 667 COUMATE might inhibit the enzyme, while being transported in rbcs. Both these sulfamates were found to inhibit CA II activity with similar potencies to that of Acetazolamide, a known inhibitor of CA II[191] and the X-ray crystal structure for the adduct of human CAII with EMATE has been resolved.[192] Since CAs are highly expressed in several tumours and may have a role in supporting their growth, it is conceivable that EMATE and 667COUMATE act *in vivo* by other mechanisms in addition to STS

inhibition. It would be interesting to see if our compounds act in a similar fashion on CA and **43** is currently being evaluated for its activity against CAII.

2.5 Recent advances and further discussion

2.5.1 X-ray crystallography of STS

At the time of writing, the crystal structure of the human steroid sulfatase had not been resolved and it is only very recently that the data have been published and made available from the Protein Data Bank (PDB).[193] In 2003, Hernandez-Guzman reported the crystal structure of the full-length active STS, purified from human placenta, as determined by X-ray crystallography at 2.60 Å resolution.[120] Elucidation of this crystal structure represents a major breakthrough in the area as scientists have been trying to isolate and purify this membrane bound enzyme for many years. Study of the three dimensional structure with molecular modelling tools should now help understand the interactions involved at the active site and assist in the future design of potent inhibitors.

As described by the authors, the tertiary structure of the enzyme is a 'mushroom-like' shape and can be divided into two domains (Figure 2.14): *(a)* a polar domain that contains the catalytic site; it resembles in shape, size and fold the two known structures of ASA and ASB; *(b)* a putative transmembrane domain that consists of two antiparallel hydrophobic α-helices, probably inserted into the lipid bilayer of the endoplasmic reticulum where the enzyme is bound. The catalytic site, which is buried deep in a cavity, seems to be located near the membrane surface, suggesting a role for the lipid bilayer in catalysis. Substrates and products may pass the membrane before and after catalysis via a hydrophobic tunnel created by the transmembrane helices. Three flexible loops, that may open to allow a steroid to enter or exit the active site, have also been identified. Figure 2.14 depicts the potent inhibitor EMATE docked into the active site of STS.

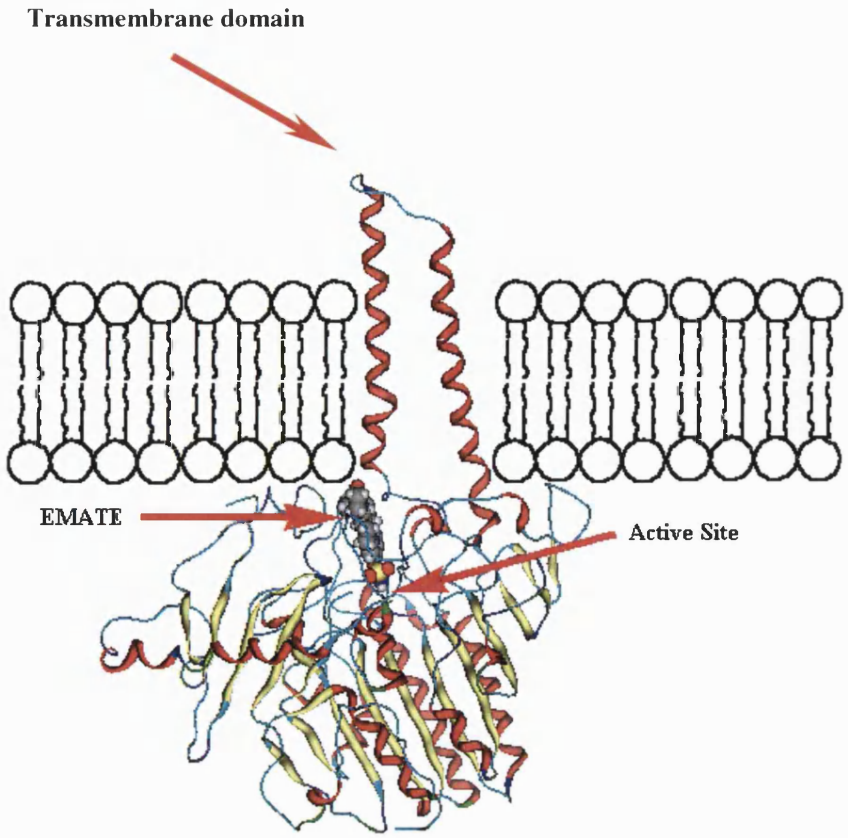


Figure 2.14 Diagram of the crystal structure of STS in association with the lipid bilayer. Adapted from Hernandez-Guzman *et al.*[120] EMATE was docked as a ligand using Gold[194] v2.1.

In the active site, the catalytic FGly is covalently linked to a sulfate moiety. This was also observed in the crystals of ASB, although it has been proposed that this sulfated derivative of FGly could be in equilibrium with its free form.[112] The most likely cation present in the reported structures of STS and ASB is Ca^{2+}, although Mn^{2+} and Mg^{2+} are possibilities that cannot be completely excluded. Most of the catalytic residues are conserved among the three mammalian sulfatases, suggesting that the mechanism of sulfate hydrolysis by human STS is similar to that previously proposed for ASB.[118]

2.5.2 Docking studies and QSAR analysis

In an attempt to rationalise the activities observed for the compounds synthesised and to gain some insight into their interaction at the active site, molecular modelling studies were carried out in collaboration with Mr J.J. Robinson, using the crystal

structure of the human STS. The use of docking algorithms can provide useful information regarding the preferred binding site of ligands in a protein. This can help identify and decide which feature(s) of a molecule give rise to its activity (i.e. pharmacophore identification) and assist in the design of compounds with enhanced properties. Quantitative structure-activity relationship (QSAR), where numerical properties of a set of molecules are related to their activity via a mathematical model can be used to predict the activity of compounds which have not been tested or synthesised as yet.

i) Docking studies

Docking methods can be used to predict energetically favourable conformations and orientations of ligands in the interior structure of a protein. These methods combine algorithms to generate different poses of a ligand (docking) and scoring functions to consider the tightness of protein-ligand interaction. GOLD[194] (Genetic Optimisation for Ligand Docking) is an automated docking program that uses a genetic algorithm to explore the full range of ligand conformational flexibility with partial flexibility of the protein. Once a conformation of minimum energy has been determined for each ligand-protein complex, the ligands can be ranked using a scoring function. The fitness score, which represents the protein-ligand interaction energy, is taken as the negative of the sum of the component energy terms, so that larger fitness scores represent higher affinity binding.

The crystal structure used for the study was obtained from the Protein Data Bank[193] and has the code 1P49. The PDB file deposited corresponds to that of the enzyme bound to a sulfate moiety which, along with all the water molecules, was removed from the structure. No energy minimisation was performed on the enzyme. For each of the compounds, 50 independent GOLD runs were performed and the best docking scores were selected. The GOLD fitness scores (GOLDScores) are presented in Table 2.6 as well as the IC_{50}s of each compound for STS inhibition in placental microsomes.

Table 2.6 STS inhibition data compared with docking scores for compounds **32-39**, **41-45** and EMATE.

Compound	IC_{50} (nM)	GOLDScore	Compound	IC_{50} (nM)	GOLDScore
32	20	60.07	**39**	12	67.87
33	12	60.92	**41**	74	65.26
34	52	61.71	**42**	23	69.10
35	1	63.20	**43**	1	67.53
36	382	64.13	**44**	3	67.78
37	150	65.38	**45**	75	63.92
38	288	68.76	EMATE	18	57.96

Each compound gave a large positive docking score, within the same range. This suggests that the D-ring modified sulfamates synthesised fit well in the active site of STS, with scores similar to that of EMATE. Since these scores vary with the physicochemistry of protein-ligand interactions, they do not necessarily correlate with the activity observed for the inhibitors, although the values obtained indicate that compounds belonging to this series should be active.

All the compounds docked so that the atoms of the sulfamate moiety available for ionic interaction are within coordination distances of Ca^{2+}. The positioning of the sulfamate group, pointing towards the central Ca^{2+} ion, dictates one major binding mode for the 14 compounds docked (Figure 2.15A and 2.15B). The steroid backbone is surrounded by mostly hydrophobic residues and the sides chains coming off the D-ring point toward the highly hydrophobic transmembrane domain (Figure 2.15A). The location of the active site, close to the membrane, might require passage of the ligands through a hydrophobic tunnel formed by the hydrophobic helices, which could explain the importance of hydrophobicity in substrate recognition and therefore enzyme inactivation.

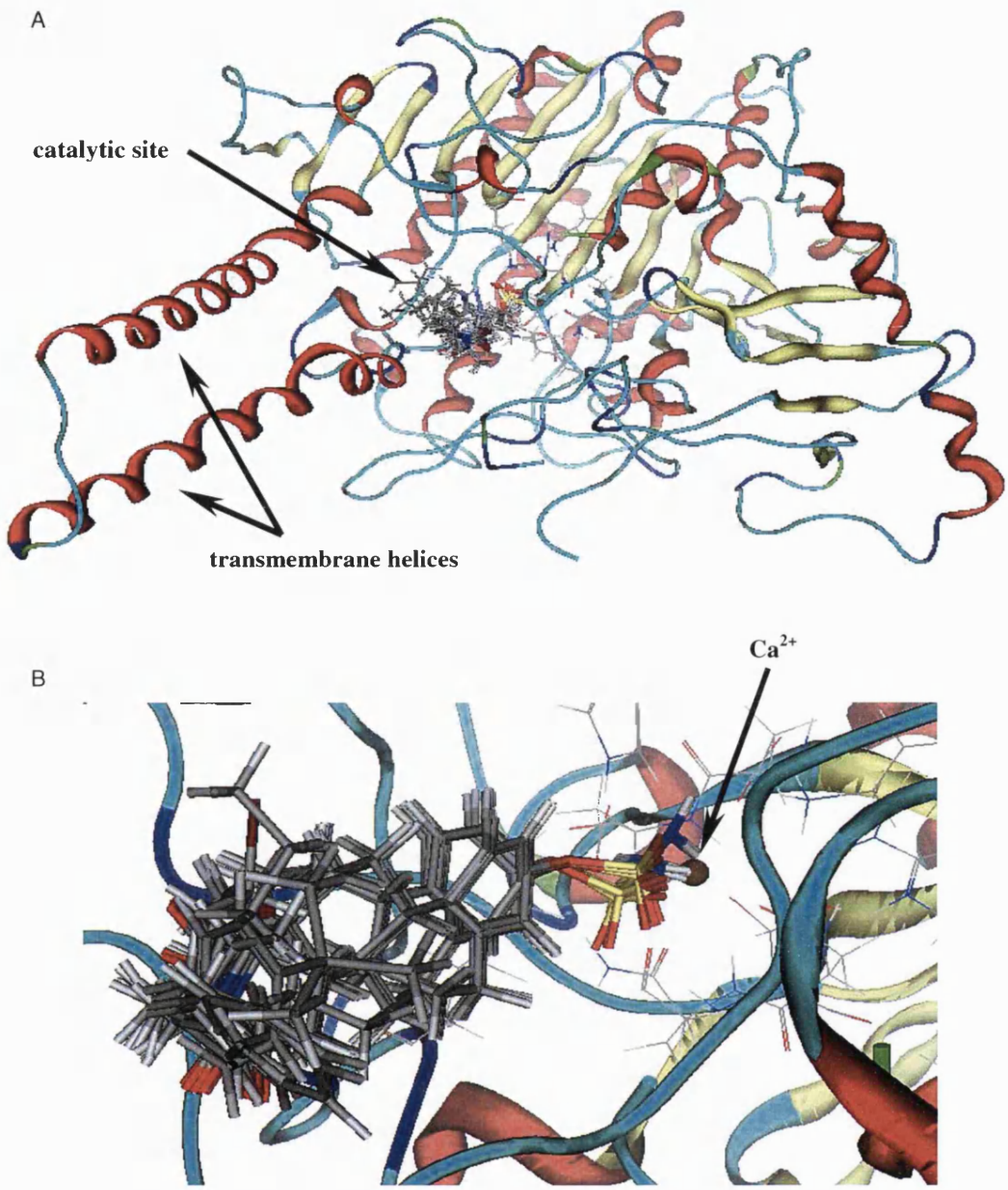


Figure 2.15 A, Docking results of the highest score solution for **32**-**39**, **41**-**45** and EMATE, shown in the tertiary structure of human STS. B, Close-up view of the active site with the docked structures and coordination of the sulfamate moieties to Ca^{2+}.

In agreement with previous reports,[195] the interaction of the sulfamate moiety with key residues in the active site and the metal ion Ca^{2+} is dominant. In their study on non steroidal phosphate esters as potential inhibitors of STS, Anderson *et al.* suggested that tight binding of a compound in the active site did not require a steroid nucleus.[195] The only essential molecular determinant important for binding appears to be the A-ring of the steroidal nucleus and an appropriately positioned charged group. Such findings contrast with other published studies, where hydrophobic interactions with the entire skeleton are required for optimal inhibition of sulfatase activity.[86,196] Both views however indicate the necessity for a negatively charged group for tight binding, which none of our inhibitors or EMATE possess. This issue was addressed by Howarth *et al.* in a docking study on estrone-3-sulfate mimics,[119] where it was postulated that the presence of a potentially cleavable group in EMATE can compensate for the lack of a charged moiety.

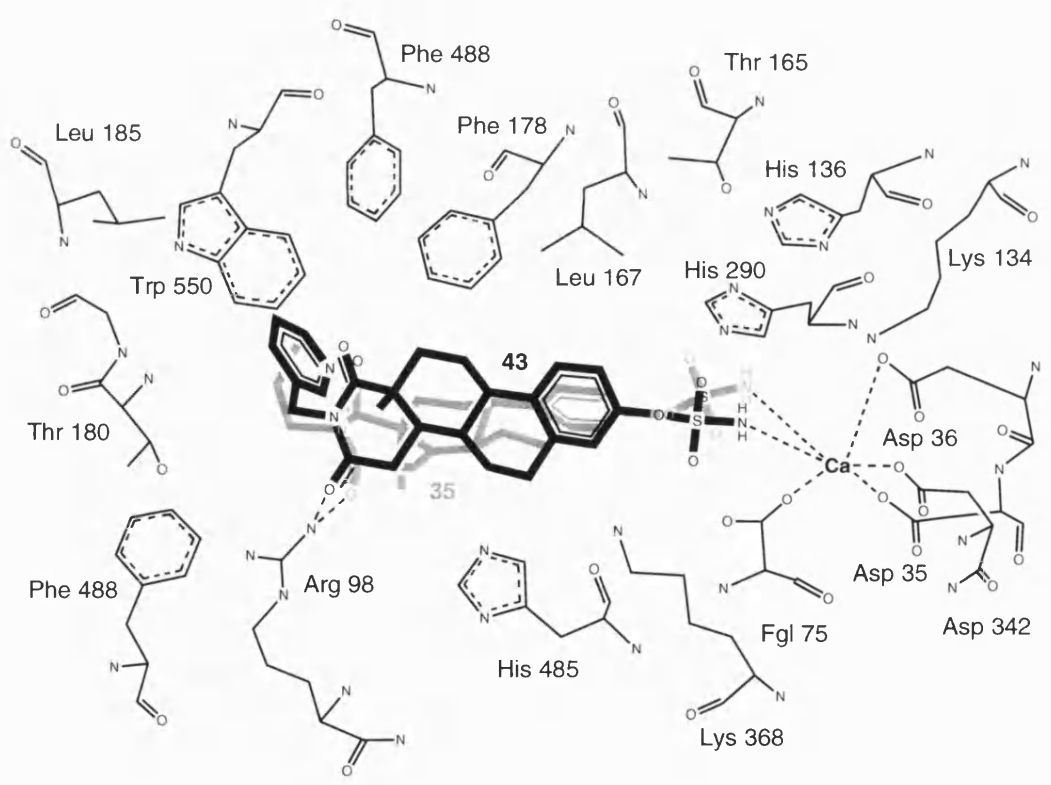


Figure 2.16 Flat projection of **35** and **43** docked in the active site of STS with surrounding amino acid residues. Dotted lines represent hydrogen bonding.

In an attempt to rationalise the high activities observed for **35** and **43**, we examined the residues around the D-ring and the side-chains of these two inhibitors docked in the active site of STS (Figure 2.16). Each inhibitor interacts in the active site via *(a)* the NH_2 group of the sulfamate moiety and the metal ion *(b)* the imide carbonyl at C16 and Arg98. Surprisingly, the imide carbonyl at C17 does not appear to participate in hydrogen bonding with catalytic residues, although such interactions could also be relayed by surrounding molecules of water (which have been removed prior to docking). The D-ring of each inhibitor is surrounded by hydrophobic residues (Leu, Phe, Trp) which illustrates previous reports[98-100,102] and our findings that hydrophobic interactions are favoured in this region. It is possible that Trp550 interacts with the pyridyl moiety of **43** via pi-pi interaction. This would in turn explain the high activity observed for the benzyl derivatives **42** and **44**.

ii) QSAR analysis

The QSAR equation uses a series of parameters to describe the properties of a molecule, which are then correlated to its biological activity. Generally, most of the parameters (or descriptors) are designed to represent the hydrophobic, electronic or steric characteristics of a molecule. The most widely used technique for deriving QSAR equations is multiple linear regression, which uses least-squares fitting, to find the best combination of coefficients for the equation.[197] To achieve statistically significant results, there should be at least five compounds for each parameter included in the regression analysis. In our case, the number of samples (14 compounds including EMATE) is too limited and this method could not be used.

Partial least squares (PLS) is a statistical method in which the original variables are replaced by a small set of their linear combinations. It is an extension of multiple regression analysis which is particularly useful in cases in which the number of descriptors is higher than the number of samples. PLS enables the use of many descriptors and still remains predictive.

Docked structures were used and several QSARs were derived using different sets of descriptors. The best relationship (Equation 1) had a correlation coefficient (R^2)

value of 0.9995 (Figure 2.17). The descriptors used to build this equation take into account the polar character of each molecule (Van der Waals surface area and volume), its hydrophobicity (logP value) and its hydrogen bond donor potential, as well as physical properties such as its weight or number of atoms.

QSAR prediction = - 4589.92 + 3.60985 PEOE-VSA-POL + 3.6628 SlogP-VSA5
- 5.45559 Weight - 19.3155 a-count + 5417.6 Petitjean + 15.9715 Vol
- 4.31349 VSA-DON - 0.423702 Weiner Path (1)

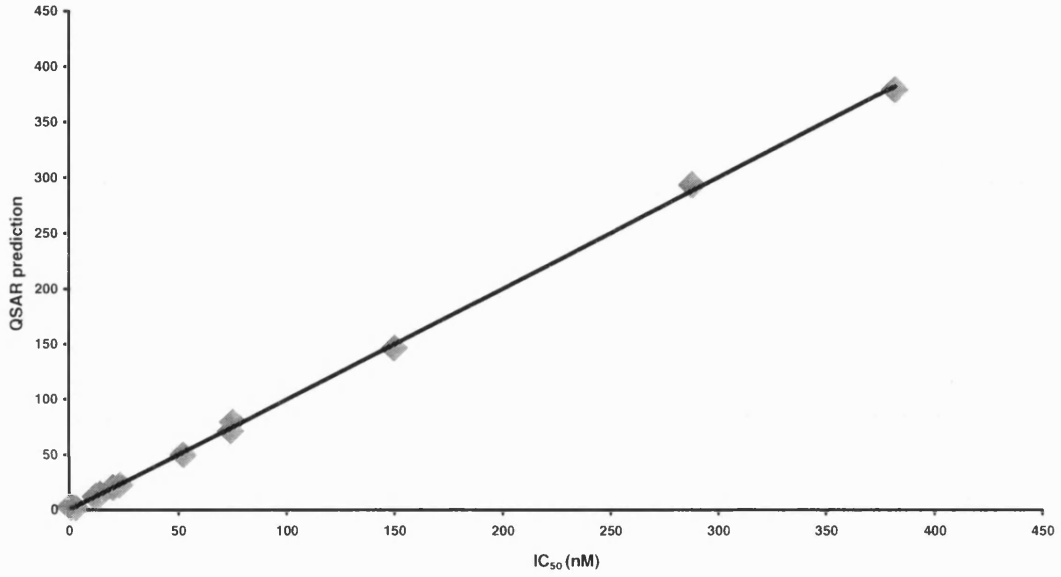


Figure 2.17 Plot of the QSAR prediction vs. IC_{50} for each compound. The equation of the trend line is $y = 0.9995x + 0.0502$; the correlation coefficients are $R^2 = 0.9995$ and $Q^2 = 0.9213$.

The predicting potential of the final QSAR equation was evaluated by the Leave-One-Out (LOO) method. Using LOO, each compound of the list was deleted once from the data set and the regression equation thereby obtained was used to predict the activity of the deleted compound. The cross validated correlation coefficient Q^2 is therefore a measure of the 'wellness' of the prediction.

Table 2.7 Experimental vs. correlated (QSAR) IC_{50} values for **32-39**, **41-45** and EMATE determined using LOO.

Compound	IC_{50} (nM)	QSAR-IC_{50} (nM)	Compound	IC_{50} (nM)	QSAR-IC_{50} (nM)
32	20	20.0092	**39**	12	12.1566
33	12	12.0069	**41**	74	71.2865
34	52	49.9671	**42**	23	22.4933
35	1	3.0043	**43**	1	2.1707
36	382	379.0415	**44**	3	1.4218
37	150	146.3974	**45**	75	79.5856
38	288	293.4210	EMATE	18	14.1761

With a Q^2 value of 0.9213, the predictive power of the QSAR equation was confirmed, as shown by the clear trend obtained in Figure 2.17. LOO predicted IC_{50}s, as well as experimentally determined IC_{50}s are given in Table 2.7 for comparison.

2.6 Conclusion

We have successfully designed a template for the rapid generation of potent STS inhibitors. Starting from EMATE, with the aim to reduce its intrinsic estrogenicity while retaining its activity, we introduced crucial modifications into the D-ring. The resulting 16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide skeleton proved to be a valuable template for the introduction of a variety of side-chains on the D-ring, that allowed us to probe the STS active site. The importance of the hydrophobicity of the D-ring moieties (at C16/C17 on E2 or EMATE) is well documented[98-100,102] and it was envisaged to explore the capacity of the potential corresponding hydrophobic pocket in the active site.

Of the analogues synthesised, three compounds were found to be highly active *in vitro* against STS, namely the propyl **35**, the pyridin-3-ylmethyl **43** and the benzyl **44** derivatives. In particular, **35** and **43** were 18 times more potent than EMATE, with IC_{50}s of 1 nM in placental microsomes. While hydrophobic residues seemed to contribute to the high activity observed, steric bulk had a major effect and long linear

alkyl side-chains led to steric clashes. Both **35** and **43** were also active *in vivo* when tested orally on a 5 day course, at 10 mg/kg/day. Their full potential as future drug candidates for estrogen-dependent diseases was confirmed when they were found to inhibit liver sulfatase activity by 99%, with no residual estrogenic activity at this dose. Metabolic studies might however be necessary to confirm the bioavailability of such inhibitors. Like EMATE, **35** and **44** might be transported by rbcs *in vivo* and thus may not undergo metabolism before they reach their target. At present, we do not have the direct proof that our inhibitors act via an irreversible mechanism, but it is believed that the A-ring pharmacophore might act synergistically with the D-ring to enhance their potency. All sulfamates so far studied are assumed to act irreversibly by way of established precedent and we do not anticipate that these will be any different.

In order to rationalise the activity of this series of inhibitors, molecular modelling studies were carried out using the recently published crystal structure of the human STS.[120] Docking experiments identified one major binding mode for all the compounds, illustrating the importance of the sulfamate moiety for binding, as reported in the literature.[119,195] Key interactions that may be involved in stabilising the inhibitor-enzyme complex have also been identified and evidence for a hydrophobic pocket in the D-ring region confirmed. A QSAR was established on the basis of the hydrophobic and polar character of the molecules as well as their physical properties. Its predictive power was confirmed and it may therefore be used to assist in the design of future inhibitors within this series.

- 3 -

17β-Hydroxysteroid dehydrogenase inhibitors

Foreword: Isotypes and selectivity

The 17β-hydroxysteroid dehydrogenases are an important class of steroidogenic enzymes that are involved in the regulation of steroid hormones such as estrogens and androgens.[127,128] Several isotypes which differ in substrate specificity, catalytic direction (oxidation or reduction) and tissue distribution have been identified.[125] Previous investigations have mainly focused on 17β-HSD type 1 and type 2, the enzymes responsible for the interconversion of E1 and E2. In particular, 17β-HSD type 1 the isoenzyme responsible for the production of the potent estrogen E2 represents an attractive target for the treatment of estrogen-dependent pathologies, such as HDBC.

Contrary to isotype 1, 17β-HSD type 2 catalyses preferentially the oxidative process (E2 to the less estrogenic E1) thereby reducing exposure of tissues to estrogens.[122,123] Inhibition of 17β-HSD type 2 might result in an increase in the intracellular level of E2 and therefore it is important to achieve selectivity when inhibiting 17β-HSD type 1. Both 17β-HSD type 1 and type 2 accept estrogenic as well as androgenic substrates, although 17β-HSD type 2 is less specific for C18 steroids than the isotype 1. To design compounds that have little or no affinity for other 17β-HSDs, in particular for 17β-HSD type 2, it is crucial to identify the molecular determinant responsible for substrate specificity. Unfortunately, the three dimensional structure of 17β-HSD type 2 remains unknown and investigation of the topology of the active site have been carried out via the design of inhibitors.[198,199]

All the potential 17β-HSD type 1 inhibitors synthesised as part of this work have therefore been tested against both isotypes 1 and 2. The inhibitory potency of compounds against type 2 was used as a measure of selectivity and selective 17β-HSD type 1 inhibitors were characterised by a lack of (or a weak) inhibition of 17β-HSD type 2.

3.1 Mechanism-based inhibitors of 17β-HSD type 1

3.1.1 Enzyme-generated alkylators: how and why?

Although the therapeutic value of inhibiting 17β-HSD type 1 has been known for many years, relatively few selective inhibitors have been reported in the literature.[72,121] Early work on inhibition was carried out by Covey and co-workers[131-133] in the 1980's, with the development of irreversible inhibitors. Prompted by the report that a yeast alcohol dehydrogenase was inactivated by allylic alcohols, the authors decided to investigate steroid molecules bearing an α,β-unsaturated alcohol moiety as potential suicide substrates for 17β-HSD type 1. Given that 17β-HSD type 1 has a bidirectional activity, the allylic alcohol functionality (latent Michael acceptor) can be enzymically oxidised in the active site to an α,β-unsaturated ketone (active Michael acceptor or affinity alkylator) which can then undergo 1,4-addition of a nucleophilic amino acid residue to form covalently inactivated enzyme (Figure 3.1).

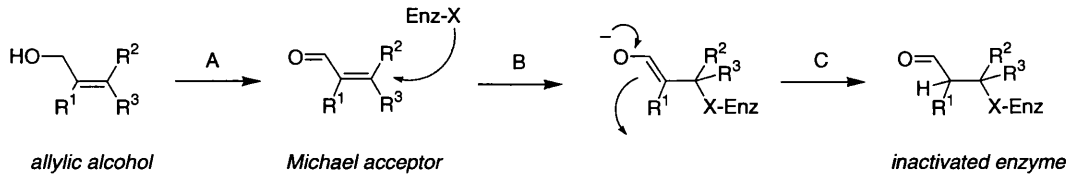


Figure 3.1 Irreversible inhibition of 17β-HSD type 1 by enzyme-generated Michael acceptor. (A) enzymatic oxidation; (B) attack by a nucleophilic amino acid and formation of an adduct; (C) proton abstraction. (Enz-X = nucleophilic residue of the active site; R1, R2 and R3 = any groups of the substrate).

Enzyme-generated alkylators represent an appealing concept for the development of selective inhibitors of 17β-HSD type 1 and have an increased specificity over irreversible inhibitors or affinity labelling agents. Conceivably, Michael acceptors can be attacked by any available nucleophile within a biological system, however, this is avoided by the use of non reactive precursors, which are only converted to reactive species by a specific enzyme. Additionally, since generation of the affinity alkylator occurs within the enzyme active site, specific alkylation of active site

amino acid residues is favoured. Finally, as one molecule of steroid is expected to remain bound to each sub-unit of the enzyme, *de novo* synthesis is required for the enzyme to recover its activity.[129]

Introduction of an α,β-unsaturated alcohol at C20, C17 or pseudo C17 of a steroid nucleus resulted in the suicide inhibitors depicted in Figure 3.2 (**1.15, 1.16** and **65**) that can potentially be oxidised to the corresponding enones (**3.1**, **3.2** and **63**, Figure 3.2) by 17β-HSD type 1. Both **1.15** and **1.16** were found to cause time-dependent inactivation of the enzyme in a concentration-dependent manner, and enzyme inhibition occurred only in the presence of a cofactor, indicating that turnover to the corresponding ketone was essential.[130,132] Unfortunately, these compounds were poor substrates for the enzyme and had a weak oxidation rate; the half-life of **1.15** was 500 min at 50 μM and **1.16** was oxidised 400 times slower than E2.

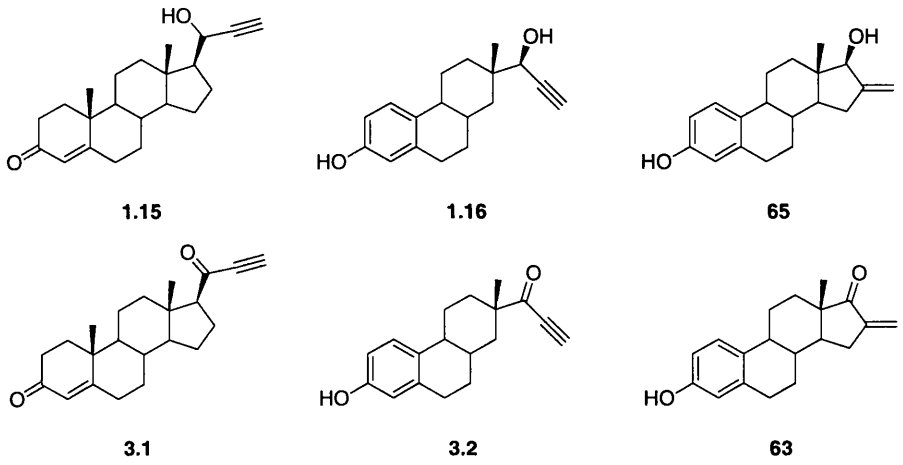
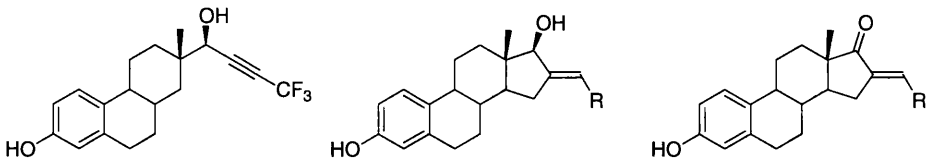


Figure 3.2 Latent (**1.15**, **1.16**, **65**) and active (**3.1**, **3.2**, **63**) Michael acceptors as irreversible inhibitors of 17β-HSD type 1.

The most promising compound was found to be 16-methylene-estradiol (**65**, Figure 3.2) since it could be oxidised at about the same rate as the normal substrate. The low reactivity of the enzyme-generated product (**63**, Figure 3.2) was however problematic. When the acetylenic derivatives were tested in their active form (**3.1** and **3.2**, Figure 3.2), the enzyme was rapidly inactivated (limiting $t_{1/2}$, <1 min for **3.2**), which suggested that, contrary to **63**, the electrophilic moiety generated *in situ* had sufficient reactivity towards residues of the active site. The high reactivity of **3.2**

toward the active site was exploited to study the structure of enzyme-steroid adducts. Using solution- and solid-state ^{13}C NMR, Auchus and Covey[200,201] identified cysteine and lysine Michael adducts after incubation of the enzyme with ^{13}C-enriched **3.2**.



3.3 **3.4** **3.5**

Figure 3.3 Structure of the affinity marker **3.3** and general structure for 17β-HSD type 1 inhibitors developed in our laboratories (**3.4** and **3.5**, R = aryl, 2-, 3- or 4-pyridyl).

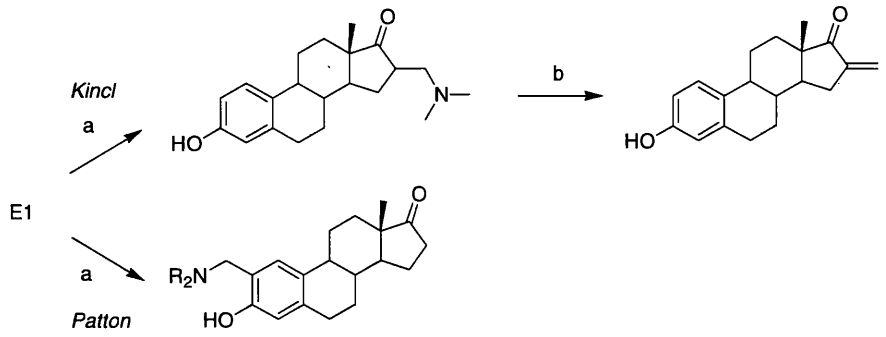
In an attempt to optimise the oxidation rate and/or reactivity of these inhibitors, a trifluoroacetylenic alcohol derivative of secoestradiol was prepared (**3.3**, Figure 3.3).[133] Unfortunately, it was proven to be reactive enough to inactivate the enzyme without the oxidation step, and behaved therefore as an affinity alkylator.

To the best of our knowledge, no new enzyme-generated irreversible inhibitor of 17β-HSD type 1 has been reported in the literature since these studies. Our interest in such compounds arose when some derivatives of E2 and E1 (**3.4** and **3.5**, Figure 3.3) developed in our laboratories displayed interesting biological activity. These compounds were found to inhibit 17β-HSD type 1 activity in T-47D cells, and in particular one E2 derivative (**3.4**, R = 4-pyridyl, Figure 3.3) had an IC_{50} of 4.1 μM. However, the mechanism through which these compounds exert their effect was not investigated and whether the E2 derivatives could be substrates for the enzyme remains unclear. Interestingly, there is no precedent in the study of 16-alkylidene derivatives of E1/E2 as inhibitors of 17β-HSD type 1, which prompted us to investigate their potential. Therefore, we decided to synthesise 16-alkylidene derivatives of E1/E2 bearing small hydrophobic moieties at C16, and in a preliminary study we aimed to prepare the propylidene, isobutylidene and dimethyl-propylidene derivatives of E1 and E2. To the best of our knowledge, such derivatives of E1 and E2 have not been synthesised before.

3.1.2 Synthesis

i) Synthesis of the 16-methylene derivatives 63 and 65

In order to test the newly synthesised compounds against a reference in the enzymatic assay, we first prepared the 16-methylene derivatives of E1 and E2 (**63** and **65**, Figure 3.2). α-Methylene ketones can be prepared by direct methylene transfer, via a Mannich reaction followed by elimination of an amine. Most of the procedures involve the use of dimethylamine hydrochloride and formaldehyde in an aprotic solvent.

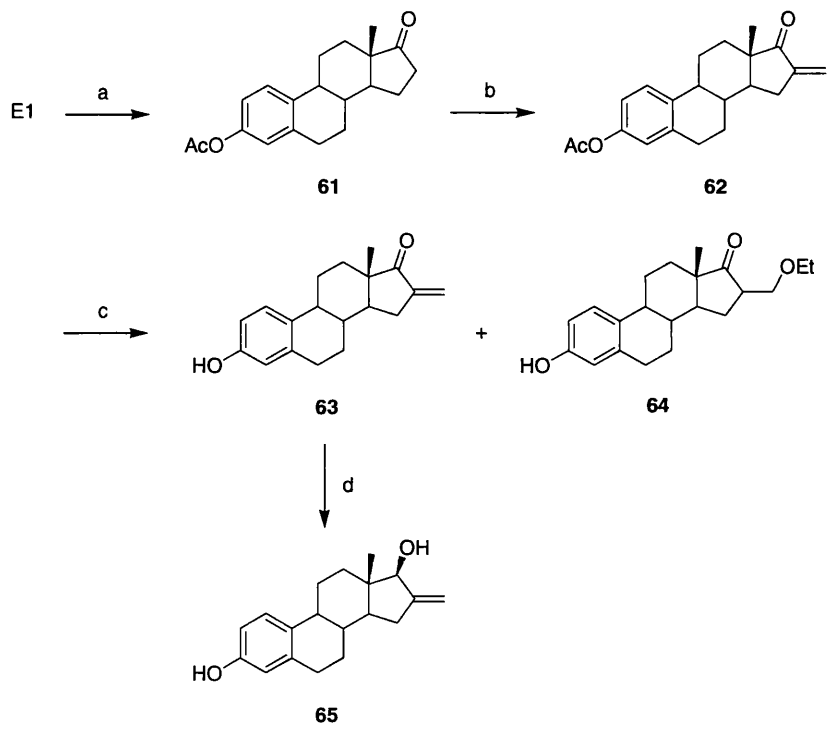


Scheme 3.1 The Mannich reaction on E1 as reported by Kincl and Garcia:[202] (a) (HCHO)n, $HNMe_2$.HCl, isoamyl alcohol, reflux; (b) steam distillation, and as reported by Patton:[203] (a) (HCHO)n, HNR_2, benzene, EtOH, reflux.

The Mannich reaction on E1 was first studied by Kincl and Garcia.[202,204] Using the above mentioned conditions in refluxing isoamyl alcohol, the reaction was reported to yield a 16-dimethylaminomethyl intermediate (Mannich base), which was then converted into 16-methylene-estrone in good yields (Scheme 3.1). Shortly after Kincl and Garcia's report, Patton[203] suggested that the Mannich base could be formed by reaction on the A-ring of estrogens. This was substantiated by a series of experiments where E1 or E2 were exclusively converted to their corresponding 2-dialkylaminomethyl derivatives under Mannich conditions in a refluxing mixture of benzene and EtOH (Scheme 3.1). Later, Gonzalez *et al.* used this method (i.e. Patton's variation) to prepare a 2-dimethylaminomethyl derivative of E2.[205] The contrast in the products obtained in Kincl's or Patton's variation may be attributed to

the difference in the solvents used, however we felt it necessary to ensure that methylenation occurred at C16 when repeating the procedure of Kincl and Garcia.[202]

A preliminary attempt to apply Kincl and Garcia's conditions to E1 failed to yield the expected product, and protection at C3 was envisaged. Acetylation of the phenolic function of E1 was achieved in quantitative yields following a literature procedure (Scheme 3.2).[156] The resulting compound **61** was then reacted with a large excess of dimethylamine hydrochloride and paraformaldehyde in refluxing isoamyl alcohol. After an acidic workup, the remaining isoamyl alcohol was distilled off using a Kugelrohr and purification of the residue by flash chromatography afforded **62** in 51% yield. Some deprotected product **63** was also recovered from the column (less than 10%), accounting partially for the moderate yield of **62**.



Scheme 3.2 Synthesis of the 16-methylene derivative of E1 and E2 **63** and **65**. (a) Ac_2O/Py, reflux; (b) (HCHO)n, $HNMe_2$.HCl, isoamyl alcohol, reflux; (c) KOH/EtOH; (d) $NaBH_4/H_2O$, MeOH/THF.

The acetate protecting group was then cleaved using KOH in ethanol although it was anticipated that nucleophilic conditions might affect the enone functionality of **62** (or that of **63** after deprotection). Using a minimal excess of KOH, our first attempt successfully afforded **63** as the sole product of the reaction in 58% yield, after

stirring the reagents at room temperature for 30 minutes. When repeating the reaction at 0°C, **64** was isolated as a side-product in 13% yield along with **63** (29%). The 16-ethoxymethyl derivative **64** was formed from the 1,4-addition of an ethoxide anion to the α,β-unsaturated ketone of **62** or **63**. The absence of low field methylenic signals in the ^{1}H NMR spectrum of **64** confirmed that the methylene functionality had reacted. The presence of a triplet at δ 1.16, accompanied by two signals accounting for two protons each at δ 3.4-3.7 further asserted the structure of this side-product which was formed as a single diastereoisomer. The conditions in which **64** was formed indicate that ester hydrolysis at C3 is the favoured reaction and occurs faster than nucleophilic addition at the D-ring end. Performing the deprotection at room temperature seemed sufficient to prevent the side-reaction. Reduction of the 17-keto function of **63** was then achieved using an aqueous solution of $NaBH_4$ in MeOH/THF.[204] This conventional method for the selective reduction of the ketone of E1 afforded the corresponding 17β-hydroxy compound **65** in 48% yield after recrystallisation.

Based on its ^{1}H NMR spectrum, **62** was assigned the structure of a 16-methylene derivative. All the aromatic protons, as well as the couplings between H1-H2 and between H2-H4 could be observed (Figure 3.4).

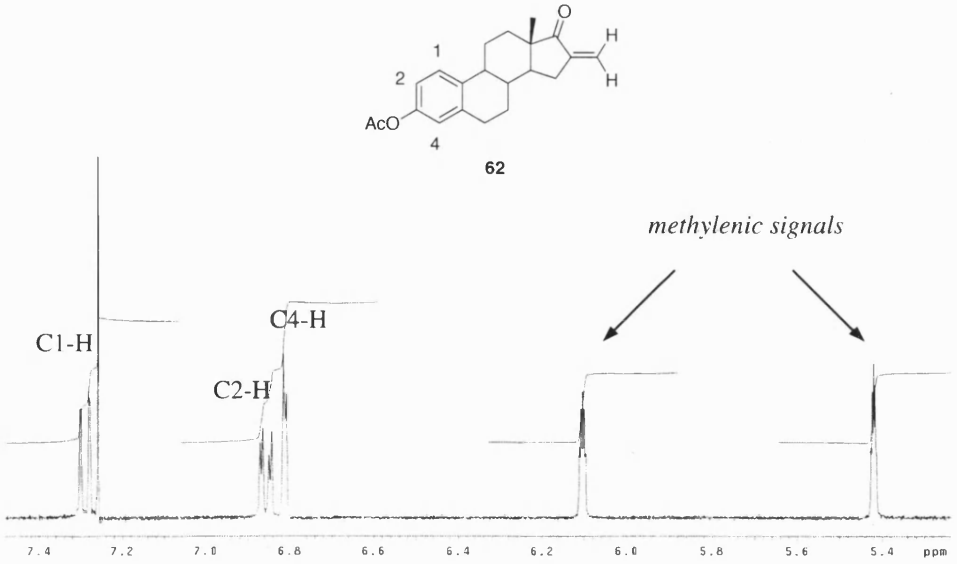


Figure 3.4 Part of the ^{1}H NMR spectrum of **62** ($CDCl_3$, 400 MHz).

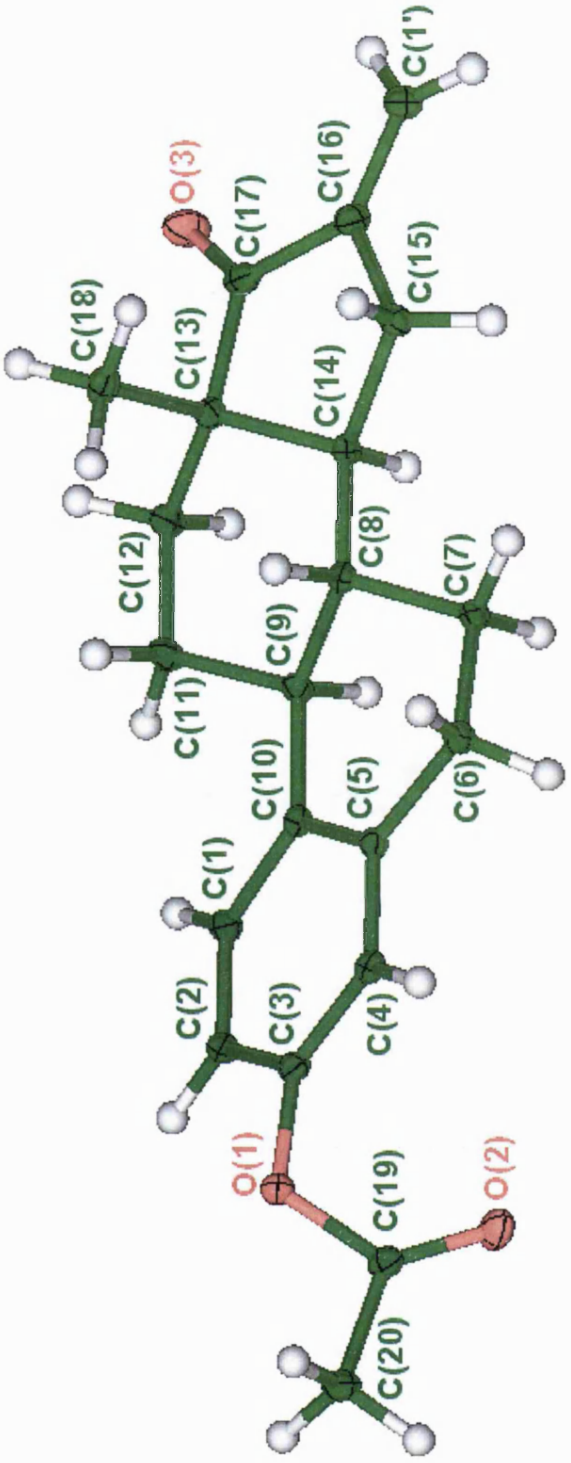


Figure 3.5 ORTEX[173] plot of the X-ray crystal structure of **62**. Ellipsoids are shown at the 30% probability level.

Unambiguous proof of this structure was later provided by X-ray crystallography. A crystal (approximate dimensions 0.35×0.28×0.25 mm) was obtained from slow crystallisation of **62** from hexane and used for data collection. The resulting ORTEX[173] plot is presented in Figure 3.5. As expected, the D-ring features an α,β-unsaturated ketone with an exocyclic methylene function at C16. Accordingly, the C=C bond has a length of 1.3245 Å while that of the conjugated carbonyl bond is of 1.2175 Å. For full details see Appendix 2. To the best of our knowledge, this is the first report of an X-ray crystal structure of an α,β-unsaturated D-ring derivative of E1. The data collected are expected to be useful for future molecular modelling studies.

ii) Synthesis of 16-alkylidene analogues

Two general methods have been described in the literature for the efficient conversion of the steroid ketone to an α,β-unsaturated moiety. The first synthetic approach involves the generation of a 16-(*N*,*N*-dimethylethylene) intermediate, which can directly be reacted with alkyllithiums or Grignard reagents to form unsaturated ketones (Figure 3.6).[205] This Vilsmeier type of intermediate can be prepared by reacting protected E1 with Bredereck's reagent[205,206] or with *N*,*N*-dimethylformamide dimethylacetal.[207] Alternatively, the enamine can be accessed in two steps via a 16-hydroxymethylene-estrone derivative followed by condensation with an alkylamine.[208]

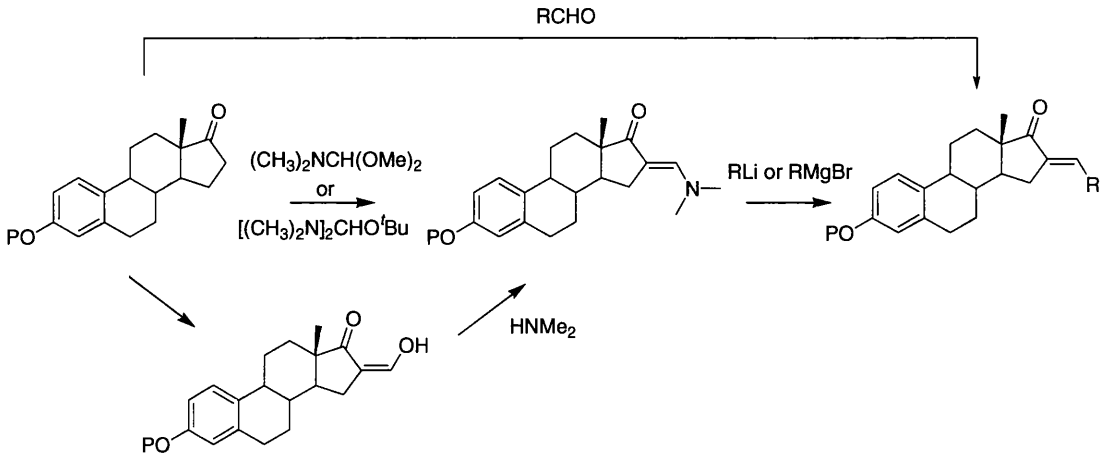
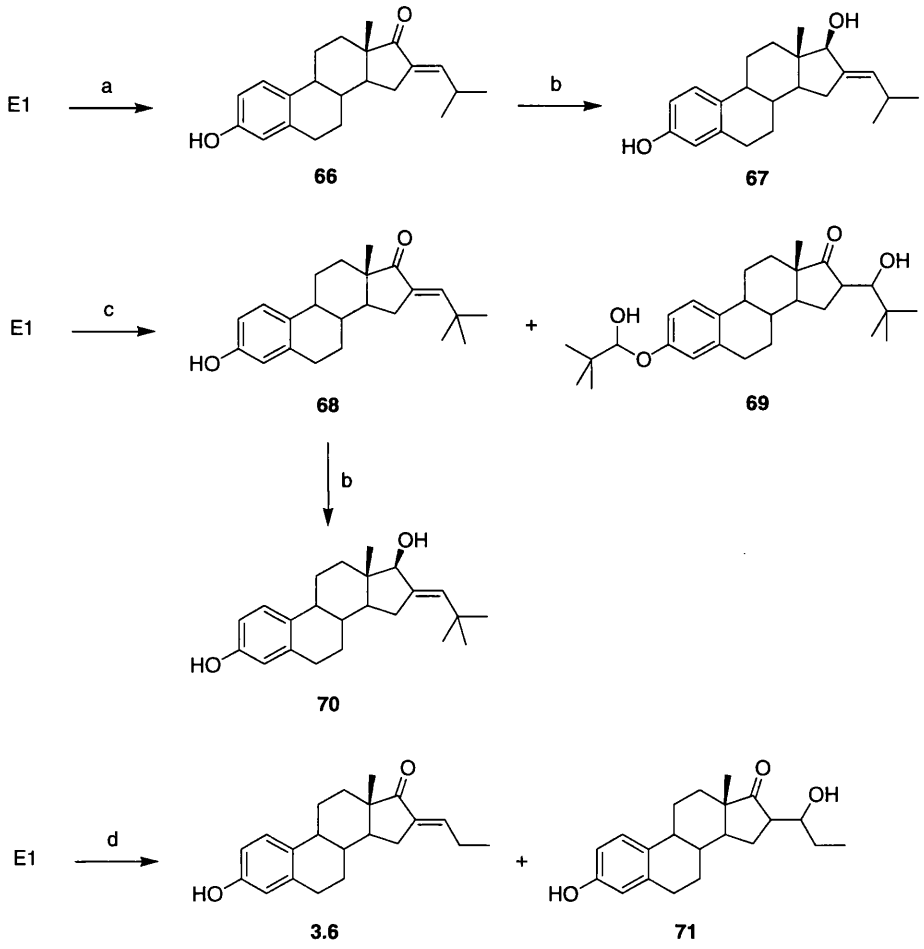


Figure 3.6 Synthetic strategies to access 16-alkylidene derivatives of E1. (Bredereck's reagent is $[(CH_3)_2N]_2CHO^tBu$; P = protecting group; R = alkyl group).

The synthesis of 16-alkylidene derivatives of E1 can also be achieved in one step via an aldol condensation between the steroid enol and an aldehyde (Figure 3.6). The cross-aldol reaction, which involves the use of aldehydes which do not possess an enolisable proton, has been successfully applied to E1 previously[164] as well as in our laboratories. Even when the aldehyde has an enolisable proton, the reaction proceeds via addition of the α-carbon of the ketone to the carbonyl of the aldehyde because of the lower reactivity of ketones vs. aldehydes towards nucleophiles. This was therefore the method of choice to access our targets.



Scheme 3.3 Synthesis of 16-alkylidene derivatives of E1. (a) LDA/THF, -78°C then $(CH_3)_2CHCHO$, -78°C to rt; (b) $NaBH_4/H_2O$, MeOH/THF; (c) LDA/THF, -78°C then ^tBuCHO, -78°C to rt; (d) LDA/THF, -78°C then CH_3CH_2CHO, -78°C to rt.

E1 was deprotonated at C16 using LDA in THF and the resulting enol was reacted with a series of aldehydes (Scheme 3.3). Condensation of E1 with isobutyraldehyde gave **66** with a yield of 79% as the sole product of the reaction. Among other peaks characteristic of the isobutylidene moiety in the ^{1}H NMR spetrum of **66**, a deshielded signal was observed at δ 6.46 for the vinylic proton. The signal for the corresponding carbon was seen at δ 144.2 in the ^{13}C NMR spectrum. Examination of the NMR spectra also suggested that one regioisomer was exclusively formed and the double bond configuration was assigned by analogy with literature reports[205,209] and was in accordance with the formation of the thermodynamically more stable *E* isomer.

The reaction of E1 with 2,2-dimethyl-propionaldehyde was found to proceed slower, possibly due to the diminished reactivity of the more hindered aldehyde, and unexpectedly resulted in the formation of two non isomeric products. After 2 days, the expected product **68** was obtained in 42% yield along with a side-product which was identified as **69** (Scheme 3.3). The presence of a peak for an additional *tert*-butyl group in the ^{1}H NMR spectrum of **69** suggested the formation of an adduct between E1 and two molecules of the aldehyde, which was confirmed by mass spectrometry. Presumably, an excess of base in the reaction mixture was able to deprotonate one hydroxyl of the intermediate aldol, which then reacted further with the aldehyde (Figure 3.7). Deprotonation of the phenol to yield **69** was most likely because of its lower pK_a (pK_a ~ 9-11) compared with that of the secondary alcohol at the D-ring end (pK_a ~ 15-19). The formation of **69**, as opposed to **3.7** (Figure 3.7), was also corroborated by the presence of a doublet of doublets at δ 3.23 which gave a doublet upon D_2O exchange in the ^{1}H NMR spectrum. Such a signal can only be seen for the proton H1″ in **69** which is coupled to C1″-OH and C16-H.

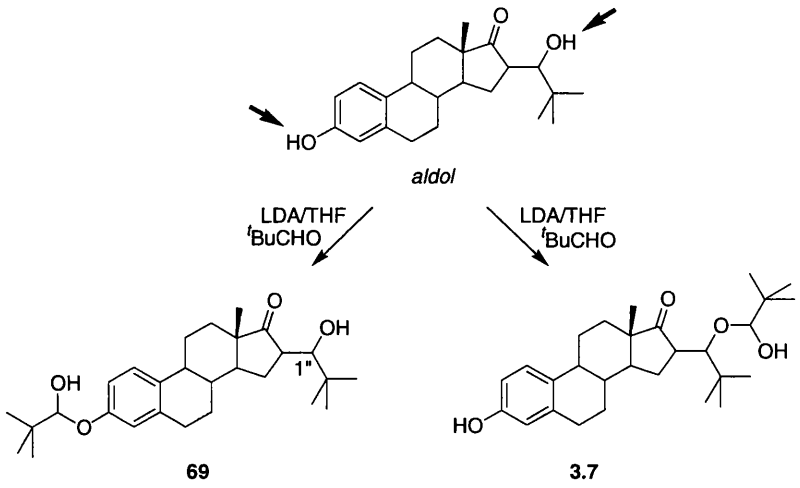


Figure 3.7 Formation of a side-product in the aldol condensation of E1 with 2,2-dimethyl-propionaldehyde. Bold arrows indicate the sites of deprotonation and condensation.

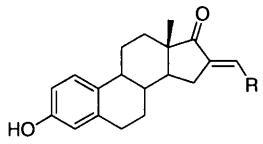
Finally, attempts to perform the condensation between E1 and propionaldehyde failed to yield the expected product (**3.6**, Scheme 3.3). Instead, the aldol **71** was obtained in 60% yield, and too little of **3.6** was recovered to allow its purification. Although the ^{1}H NMR spectrum showed **71** as a single compound, its ^{13}C NMR indicated that it was formed as an inseparable mixture of diastereoisomers. Reduction of the 17-ketones of **66** and **68** into the corresponding 17β-alcohols proceeded smoothly using the same conditions as for the reduction of **63** (cf. Scheme 3.2). The derivatives **67** and **70** were obtained in respective yields of 60% and 79% after recrystallisation.

3.1.3 Results and discussion

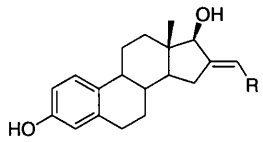
i) Inhibition of 17β-HSD type 1 in vitro

The compounds prepared were examined for their ability to inhibit 17β-HSD type 1 activity in the human hormone-dependent breast cancer cell line T-47D. For each compound, 17β-HSD type 2 activity was also assessed as a measure of selectivity of inhibition. The percentage of inhibition achieved for a concentration of 10 μM of inhibitor is given in Table 3.1 and 3.2. The 16-methylene and 16-alkylidene derivatives were evaluated in both their latent and active Michael acceptor forms.

Table 3.1 Inhibition of 17β-HSD type 1 (and type 2) by the α,β-unsaturated ketone derivatives **63**, **66**, **68** and the allylic alcohols derivatives **65**, **67** and **70**. Results are expressed as a percentage of inhibition at 10 μM (± S.D. triplicate).



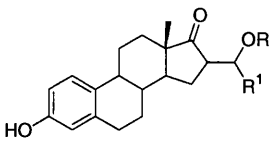
		% inhibition at 10 μM	
R	Compound	17β-HSD type 1	17β-HSD type 2
H	**63**	70.3 ± 3.8	9.7 ± 5.9
$CH(CH_3)_2$	**66**	71.4 ± 2.7	17.8 ± 4.6
$C(CH_3)_3$	**68**	81.8 ± 1.3	31.3 ± 3.3



		% inhibition at 10 μM	
R	Compound	17β-HSD type 1	17β-HSD type 2
H	**65**	63.7 ± 2.3	19.9 ± 0.7
$CH(CH_3)_2$	**67**	68.3 ± 1.4	7.1 ± 5.9
$C(CH_3)_3$	**70**	83.9 ± 2.2	27.1 ± 2.4

All the compounds tested inhibited 17β-HSD type 1 activity to more than 60% at a concentration of 10 μM and displayed selectivity for the type 1 isoenzyme, i.e. they were weak inhibitors of 17β-HSD type 2. In the α,β-unsaturated ketone series, the best inhibitor was the dimethylpropylidene derivative **68**, which achieved 82% inhibition of type 1 activity. Its corresponding allylic alcohol **70** was the best inhibitor among the latent Michael acceptors with 84% inhibition of 17β-HSD type 1 activity. Disappointingly, **63** and **65** proved to be moderate inhibitors of 17β-HSD type 1 at 10 μM, with respective levels of inhibition of 70% and 64%. The activity of **63** was however consistent with reports that this enone had low reactivity towards the active site.[131]

Table 3.2 Inhibition of 17β-HSD type 1 (and type 2) by the E1 derivatives **64** and **71**. Results are expressed as a percentage of inhibition at 10 μM (± S.D. triplicate).



			% inhibition at 10 μM	
R	R^1	Compound	17β-HSD type 1	17β-HSD type 2
Et	H	**64**	92.0 ± 0.1	19.2 ± 7.2
H	Et	**71**	79.7 ± 0.1	-17.5 ± 2.0

Both **64** and **71** were comparatively good inhibitors of 17β-HSD type 1. In particular, **64** was the best inhibitor of all the compounds tested and **71** was highly selective for the type 1 isoenzyme, with a total lack of inhibition of 17β-HSD type 2. However, these compounds do not enter the scope of the present study and their activity will be addressed in another chapter.

The α,β-unsaturated ketones and their corresponding allylic alcohols (Table 3.1) were found to be equipotent for the inhibition of 17β-HSD type 1, with the exception of **63/65**. For this pair of active and latent Michael acceptors, the level of inhibition achieved by the allylic alcohol **65** was lower than that of the enone **63**. To some extent, a difference of activity between active and latent Michael acceptors is expected since the latter needs to be oxidised before being able to irreversibly alkylate the enzyme. It is conceivable that after a short incubation time (30 minutes in this assay) the enzymatic oxidation of **65** to **63** was not complete. Using higher concentrations of **63** or **65** in the assay should afford a faster inactivation of the enzyme since the reported Km values for these compounds were 2.7 μM and 8.0 μM respectively.[131]

The moderate activities observed for the pair of inhibitors **63/65**, and in particular the lower activity of **65** might also be related to the pH at which the assay was performed (pH 7.4). For the alkylator to be generated enzymically, 17β-HSD type 1 oxidative activity needs to be sufficiently high in the assay conditions. Recent reports clearly

state that 17β-HSD type 1 has a higher specificity towards E1 reduction than E2 oxidation at physiological pH,[124,210] therefore an inefficient oxidation of **65** by the enzyme might account for its lower potency. Previous reports also indicate that a 22-fold slower inhibition of 17β-HSD type 1 at physiological pH vs. pH 9.2 was observed with **63/65**.[131] An enhanced nucleophilicity of residues such as cysteine, lysine or histidine at higher pH values was proposed to rationalise this observation. Finally, it is conceivable that the ketone **63** is reduced back to **65** faster than the inactivation occurs, accounting for the disappointing 70% inhibition at 10 μM.

Unlike **65**, it is not known whether **67** or **70** are substrates for the enzyme. Moreover, no reversibility study or kinetic data support the inhibition results, and it is therefore difficult to anticipate how these compounds inhibit 17β-HSD type 1. Four possible scenarios can be envisaged to rationalise their moderate activities:

(a) **67** and **70** are substrates for 17β-HSD type 1, but as in the case of **65**, the turnover of the oxidation reaction is too low (due to the pH, the length of the assay or the concentration of inhibitor used);

(b) **67** and **70** are substrates for 17β-HSD type 1, but as in the case of **63**, the reactivity of the Michael acceptor generated *in situ* is not very high;

(c) **67** and **70** are not substrates for 17β-HSD type 1 and the level of inhibition seen is the result of another mechanism, possibly inhibition via reversible interactions;

(d) a combination of *(a)* and *(b)*.

Upon examination of the biological results, it is not possible to conclude whether the new inhibitors **67** and **70** are actual substrates of the enzyme and whether their corresponding enones (**66** and **68**) inhibit the enzyme via an irreversible mechanism. The presence of a bulky moiety at C16 in these compounds might prevent access of the cofactor to the active site or its correct positioning for hydride transfer to occur at C17. It is however interesting to notice that if **67** and **70** do behave as enzyme-generated inhibitors, they are more potent than **65**. This might be explained by the fact that their respective α,β-unsaturated ketones **66** and **68** are better inhibitors than **63**, suggesting that the enzyme-generated products exhibit a higher reactivity towards the active site.

ii) Reversibility study on 63

Before investigating further the new inhibitors synthesised, we wanted to assess whether **63** could be used as a control or reference, and therefore whether it inhibited the enzyme via an irreversible mechanism in our assay. To this end, a reversibility study was carried out in which T-47D cells were pretreated with the inhibitor **63** (at 10 or 20 μM) overnight. 17β-HSD type 1 activity was then measured in four different cases (Figure 3.8):

(a) the cells were washed and the inhibitory activity of **63** (at 10 μM) was measured under standard assay conditions (P+W+I);

(b) the cells were not washed and the inhibitory activity of **63** (at 10 μM) was measured under standard assay conditions (P+NW+I);

(c) the cells were not washed and activity was measured in the absence of inhibitor (P+NW+S);

(d) the cells were washed and activity was measured in the absence of inhibitor (P+W+S).

A standard assay measurement was also carried out at 20 μM and Figure 3.8 summarises the results.

Clearly, a loss of inhibition can be observed upon washing the pretreated cells, whether **63** was tested at 10 or 20 μM. The remaining inhibitory activity in cells which have been pretreated but not washed (P+NW+S) is much higher than for those which undergo washing (P+W+S). Indeed, the level of inhibition falls from 57% to 35% at 10 μM and from 85% to 62% at 20 μM. This means that the recovery of the enzymatic activity upon washing is of 23%, irrespectively of the concentration of inhibitor used.

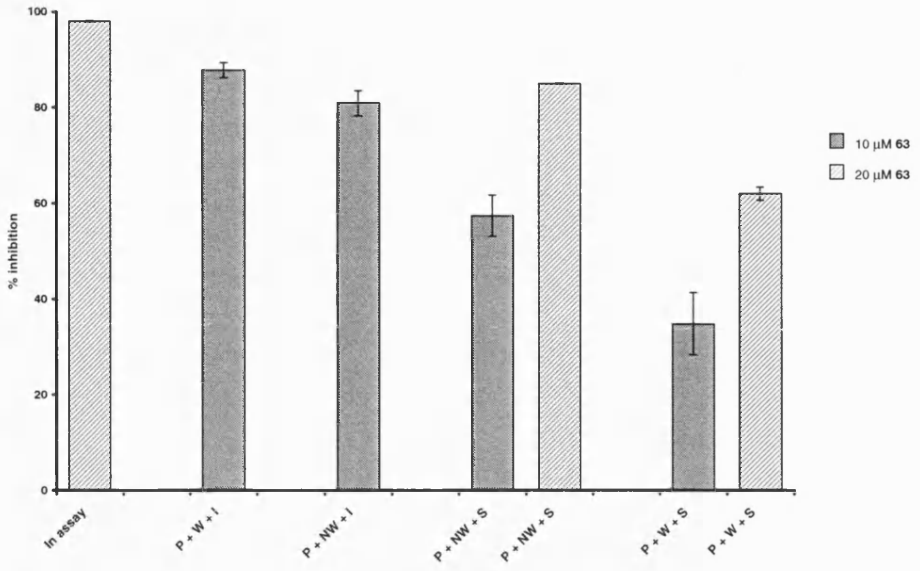


Figure 3.8 Inhibition of 17β-HSD type 1 activity in T-47D cells. Results are shown as percentage inhibition (± S.D. duplicate) of the control. The control was not pretreated with inhibitor overnight. P = pretreatment with **63** (10 µM or 20 µM) overnight; W = cells washed five times with assay medium; NW = cells not washed (assay medium removed); I = assay medium + ^{3}H-E1 + **63** (10 µM) followed by 30 minutes incubation; S = assay medium + ^{3}H-E1 followed by 30 minutes incubation; In assay = inhibition under standard assay conditions, i.e. without overnight pretreatment, after 30 minutes incubation with ^{3}H-E1 + **63** (20 µM).

As expected, washing of the cells does not significantly affect the level of inhibition when they are pretreated overnight and their activity measured in presence of the inhibitor. In both cases (P+W+I and P+NW+I) a high level of inhibition is observed (81 to 88%), with no significant difference. Maximum level of inhibition (98%) is reached when the cells are tested in presence of **63** at a concentration of 20 µM, under standard assay conditions.

Although the influence of washing seems to indicate that the inhibitor **63** may not be tightly bound to the enzyme, the recovery of enzymatic activity does not exceed 23%, which is conceivable, even for an irreversible inhibitor. To some degree, a loss in inhibition is expected upon washing the cells as it is not possible to ascertain whether all the enzyme molecules are irreversibly inactivated the moment the washing procedure is started. As mentioned earlier, the rate of inactivation is greatly enhanced at higher pH values and in previous experiments,[131] 1 hour was necessary

for **63** (at 100 μM) to inhibit half the enzymatic activity ($t_{1/2}$) at pH 9.2 vs. 25 hours at pH 7.4. Given that our assay is carried out at physiological pH, this might explain why only 35% and 62% of inhibition are achieved for 10 and 20 μM of **63** respectively after overnight incubation and washing.

Compound **63** therefore appears to behave as an irreversible inhibitor but the conditions of the assay need to be adjusted to observe an optimal effect of this inhibitor on the enzyme after overnight pre-treatment.

3.1.4 Towards a mechanism of irreversible inhibition

Irreversible inhibition using enzyme-generated inhibitors is thought to occur by Michael addition of an enzymic nucleophile to the electrophilic moiety of the ligand. Although kinetic studies corroborate such a mechanism, there is no clear proof as to whether such an attack occurs, and if so, which amino acid(s) is(are) involved. Our aim was to investigate the active site of 17β-HSD type 1 using molecular modelling in order to identify putative nucleophilic amino acid(s) thought to be involved in irreversible inhibition by **63**.

i) Literature precedent

The first evidence in support of a nucleophile-mediated irreversible inhibition of 17β-HSD type 1 was reported by Auchus and Covey, in their study on the enzyme-generated acetylenic ketone **2** (Figure 3.9).[200]

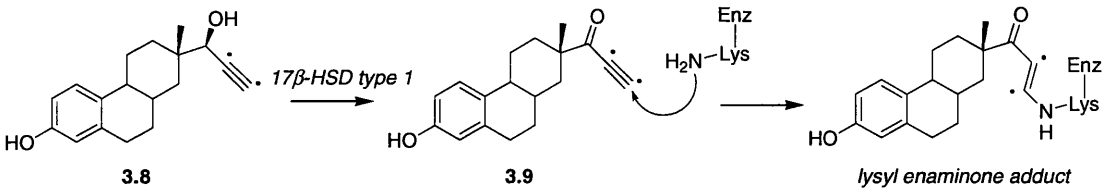


Figure 3.9 Proposed irreversible inhibition of 17β-HSD type 1 by the enzyme-generated ketone **3.9** via attack of a lysine residue (Enz-Lys-NH_2) (· = ^{13}C). Adapted from Auchus and Covey.[200]

To elucidate the structure of the potential enzyme-steroid adduct formed during irreversible inhibition, ^{13}C-enriched allylic alcohol (**3.8**, Figure 3.9) was prepared and incubated with purified 17β-HSD type 1 at pH 9.2.[200] Analysis of the inactivated enzyme by solution-state ^{13}C NMR, revealed the presence of a lysyl enaminone adduct (Figure 3.9). It is not excluded that other adducts between the steroid and the enzyme may have been formed, only they were not detected in the experiment. A follow-up study confirmed the previous findings using solid-state ^{13}C NMR.[201] From a spectra of lyophilised intact inactivated enzyme, a lysine- as well as a cysteine-Michael adducts were identified.

Although these studies have provided valuable insight into the irreversible inhibition of 17β-HSD by enzyme-generated inhibitors, they do not entirely reflect what would happen at physiological pH. In both cases the incubations were performed at pH 9.2, which may have led to an 'artificial' deprotonation of some residues such as lysine, resulting in an enhanced nucleophilicity. We believed that the use of the now available crystal structure of 17β-HSD type 1 might provide an alternative way of identifying the particular residues involved in covalent bonding with the steroid.

ii) Docking in the active site of 17β-HSD type 1

Docking studies were performed on **63** in order to predict the energetically favourable conformations of this ligand in the active site and allow the identification of key residues surrounding the steroid. The docking programme GOLD[194] and the crystal structure of 17β-HSD type 1 at 1.70 Å resolution in complex with E1, in the absence of cofactor (1 FDS[146] in the Protein Data Bank[193]) were used for this study. This particular crystal structure was chosen since a preliminary experiment using the crystal structure of 17β-HSD type 1 in complex with E2 and NADP$^+$[146] did not allow the identification of residues of the active site within proximity of the steroidal D-ring, due to the presence of the cofactor in this region. In addition, kinetic studies on the acetylenic derivative **3.9** (Figure 3.9) have shown that inactivation of the enzyme by Michael acceptors cannot occur in the ternary complex formed between the enzyme, the inhibitor and NAD.[132]

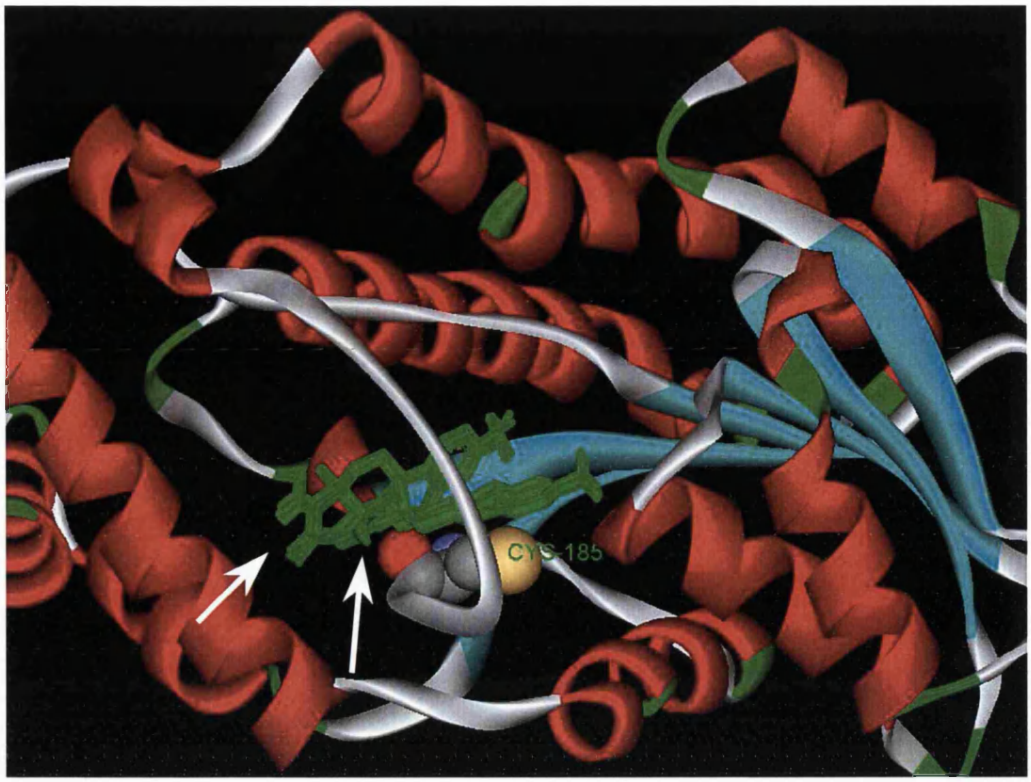
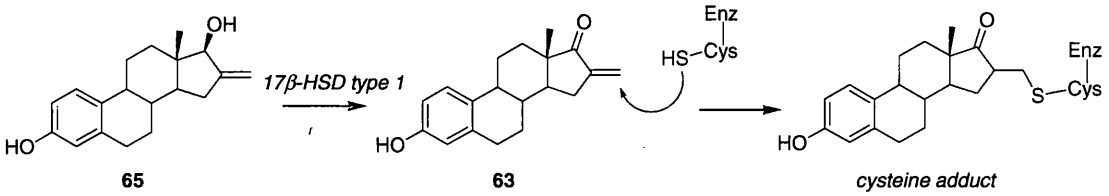


Figure 3.10 Docking solutions for **63** in the active site of 17β-HSD type 1 (1 FDS).[146] The potential nucleophilic amino acid involved in irreversible inhibition, Cys185, is shown (sulphur atom in yellow) and the white arrows point towards the D-ring.

A model of **63** was built based on the X-ray crystal structure of **62** (the 3-*O*-acetyl precursor, cf. section 3.1.2) and docking was performed using GOLD.[194] The docking modes of higher score (i.e. lower energy) obtained for **63** after 50 independent GOLD runs are shown in Figure 3.10. Clearly, two major binding modes of low energy can be identified for this steroid.

Examination of the active site within a 7 Å radius around the steroidal binding cleft identified only one candidate for nucleophilic attack on the α,β-unsaturated ketone **63**: a cysteine residue, namely Cys185 which is shown in Figure 3.10. Although Cys185 appears to be at quite a distance from the putative site of attack, it is conceivable that its side-chain comes within close contact of the steroidal D-ring as a result of conformational changes occurring in the protein. No other potential nucleophilic amino acids (His, Lys, Ser) were observed within a radius of 10 Å

around the steroid, suggesting that Cys185 is the most likely candidate for the formation of a Michael adduct with **63**. This is in agreement with the findings of Auchus *et al.*, where cysteine and lysine adducts were identified between a steroidal acetylenic ketone (**3.9**, Figure 3.9) and the enzyme.[201]



65 **63** *cysteine adduct*

Figure 3.11 Proposed irreversible inhibition of 17β-HSD type 1 by the enzyme-generated ketone **63** via attack of a cysteine residue Cys 185 (Enz-Cys-SH).

We therefore propose a putative mechanism for the irreversible inhibition of 17β-HSD type 1 by **63** involving Cys185, as depicted in Figure 3.11. The 1,4-addition of the thio-group of Cys185 onto the α,β-unsaturated system of **63** results in the covalently modified enzyme.

3.1.5 Conclusions and perspectives

In a limited study on enzyme-generated Michael acceptors as potent selective irreversible inhibitors of 17β-HSD type 1, we successfully synthesised a series of 16-alkylidene derivatives of E1 and E2.

In vitro evaluation of the new derivatives in a routine assay led to inconclusive results. The isobutylidene and dimethyl-propylidene analogues (**66/67** and **68/70**) had similar or better potency than the known 16-methylene derivatives of E1 and E2 (**63** and **65**) when tested at 10 μM, however, their mechanism of action was not elucidated. Reversibility studies indicated that **63** behaved as an irreversible inhibitor of 17β-HSD type 1, although the assay conditions clearly need to be adjusted in order to observe total inactivation of the enzyme by this inhibitor. Similar investigations on **66** and **68** should contribute to elucidating how these compounds inhibit the enzyme and further kinetic studies may help determine whether the allylic alcohols **67** and **70** are substrates for 17β-HSD type 1. Time- and concentration-

dependent curves should also help establish if the inhibitors are irreversible and competitive. Reports that inactivation was enhanced at higher pH values prompted us to investigate inhibition at pH ~ 9, and this is currently being addressed.

With the help of molecular modelling, we were able to identify a potential amino acid that might be involved in covalent binding of the steroid to the enzyme. A cysteine (Cys185) residue was the only nucleophilic candidate found in the vicinity of the steroid after docking in 17β-HSD type 1 active site and a putative mechanism of irreversible inhibition is proposed. To confirm covalent bond formation and support these data, analysis of the reacted enzyme by mass spectrometry and calculation of the molecular mass of the enzyme-inhibitor adduct would be required. Proteolytic digestion of the inactivated enzyme and analysis of the fragments should also help confirm the nature of amino acid(s) involved in the nucleophilic attack on the inhibitor.

3.2 Heterocyclic derivatives of E1

3.2.1 Identification of new lead structures

i) Background

Following the purification[211] and later the cloning of the cDNA sequence of 17β-HSD type 1,[212] the first insights into the topography of the active site and the identification of its critical residues were achieved thanks to affinity labelling studies.[131,213-215] Shortly afterwards, mechanism-based inhibitors assisted the investigations of the enzyme catalytic mechanism, producing the first active site-directed irreversible inhibitors of 17β-HSD type 1.[131-133] From these early studies, the steroidal D-ring emerged as a promising location for the introduction of structural modifications able to block the enzymatic activity and this concept was further explored by Poirier's group in the 1990's. Investigations around a series of 16α and 17α-substituted E2 derivatives showed that better inhibition was obtained for compounds bearing a good leaving group at the end of a C16α side-chain.[136] This was exemplified by the 16α-bromobutyl derivative (**1.18**, Figure 3.12), the lead compound of these studies, which had an IC_{50} of 0.46 μM in human placental cytosol.[135] It was found to irreversibly inhibit the enzyme in a time-dependent manner[136] and was later found to be an agonist of the estrogen-sensitive human breast tumour cell line ZR-75-1.[138]

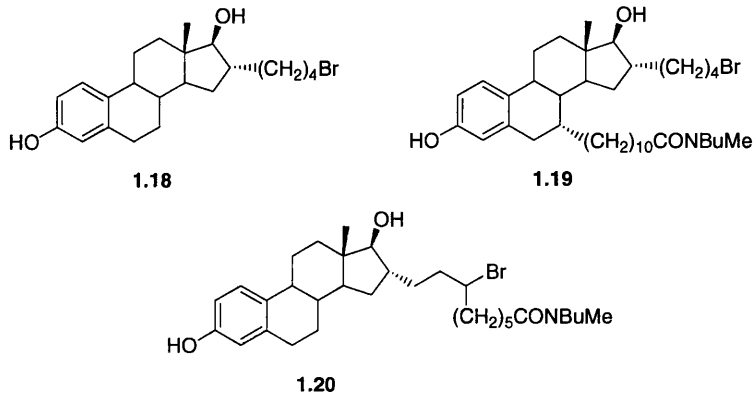


Figure 3.12 Structure of steroidal inhibitors of 17β-HSD type 1 developed by Poirier *et al.*[135,136,138]

With the growing awareness of the implication of 17β-HSD type 1 in estrogen metabolism, and, in particular, the potential benefit of inhibiting its activity to treat estrogen-dependent diseases, attention has shifted towards the design of inhibitors devoid of estrogenic activity. Having identified a potential lead, Poirier *et al.*'s strategy was to introduce the bromobutyl pharmacophore in a known antiestrogen (ICI 164,384) and in a concurrent approach, to introduce an antiestrogenic side-chain in a potent 17β-HSD type 1 inhibitor (e.g. **1.18**, Figure 3.12). Unfortunately, the bulky antiestrogenic side-chains proved to be detrimental to the inhibitory activity, and a drop of potency by at least 10-fold was observed for the resulting compounds (**1.19** and **1.20**, Figure 3.12). However, one bromoalkylamide derivative (**1.20**, Figure 3.12) had antiestrogenic properties at 1 μM.[138]

To the best of our knowledge, no lead compound has been reported since 16α-bromobutyl-estradiol (**1.18**, Figure 3.12). Given that attempts to optimise its properties were only partially successful, we wanted to identify new substrate-based structures that would inhibit 17β-HSD type 1 activity and which could potentially be modified in case of residual estrogenicity.

ii) Preliminary work, results and choice of a new template

In an initial approach to the identification of novel potent inhibitors of 17β-HSD type 1, we decided to assess the inhibitory activity of two synthetic intermediates, namely the 16-oximino and 16-hydroxymethylene derivatives of E1 (**1** and **97**, Figure 3.13). Both compounds possess small hydrophilic moieties which can potentially interact with hydrogen bond donors or acceptors in the active site. We also hoped that these compounds, which are structurally related to **69** and **72** (Figure 3.13) synthesised earlier (cf. section 3.1), would exhibit a similar level of activity.

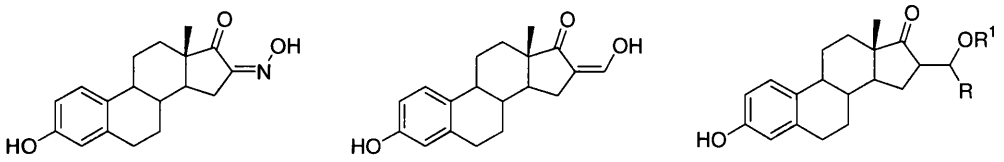


Figure 3.13 Structure of the synthetic intermediates **1** and **97** and the 17β-HSD type 1 inhibitors **64** and **71**. Compound **1**, **64** and **71** were prepared in the course of this work, while **97** was synthesised in our laboratories by Dr. C. Bubert.

When tested at 10 μM for the inhibition of 17β-HSD type 1 in T-47D cells, **1** and **97** inhibited the enzymatic activity in excess of 95% (Table 3.3). Both compounds were found to inhibit selectively the isotype 1, as they displayed low activities against type 2. With respective IC_{50} values of 1.10 μM and 0.11 μM, **1** and in particular **97** emerged as a highly potent inhibitors of 17β-HSD type 1, which further confirmed the validity of targeting the D-ring to introduce functionalities and identify potential pharmacophores.

Table 3.3 Inhibition of 17β-HSD type 1 (and type 2) activity by **1**, **97**, **64** and **71**. Results are expressed as percentage of inhibition at 10 μM (± S.D. triplicate). IC_{50} values are given as means of at least two determinations.

	% inhibition at 10 μM		IC_{50} (μM)
Compound	17β-HSD type 1	17β-HSD type 2	17β-HSD type 1
1	95.7 ± 1.0	5.0 ± 4.2	1.10
97	96.6 ± 0.2	17.4 ±1.9	0.11
64	92.0 ± 0.1	19.2 ± 7.2	0.32
71	79.7 ± 0.1	- 17.5 ± 2.0	nd

Although the geometries of C=C and C=N bonds at C16 for compounds **1** and **97** has not been investigated, it is anticipated that intramolecular hydrogen bonding could stabilise both compounds in the *cis* geometry, where a 6-membered ring system can be formed (Figure 3.14) as a result of hydroxy-keto interactions. Alternatively, the lower activity of **1** compared with **97** could be explained by its inability to form such a cyclic hydrogen bond network if the oxime exists in the *trans* geometry and

suggests that such a requirement may be crucial for activity. This is corroborated by the results obtained previously for compound **71**, where such a hydrogen bonded system can potentially be formed between the hydroxyl group of the side-chain and the carbonyl at C17. Conceivably, a pseudo 6-membered ring hydrogen bond network can also be created via the assistance of a hydrogen bond donor residue or a molecule of water in the vicinity of the D-ring (Figure 3.14), which might be the case in **64**. Therefore, it appears that compounds which are able to mimic a D-ring fused cyclic system via intra or intermolecular hydrogen bonding are powerful inhibitors of 17β-HSD type 1.

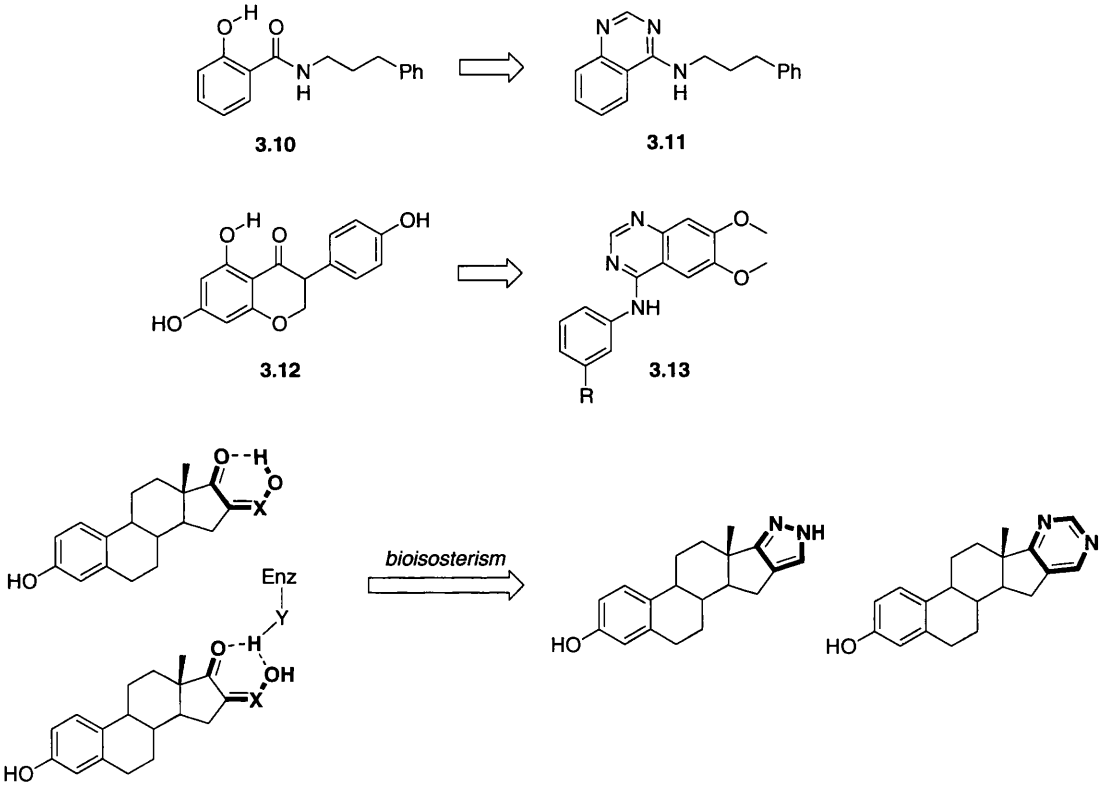


Figure 3.14 Examples of bioisosteric replacements and proposed targets to mimic cyclic hydrogen bonding in **1** (X = N) and **97** (X = C). Enz-YH is a hydrogen bond donor residue of the active site.

Heterocycles have been used in the past as bioisosteric replacements of cyclic intramolecular hydrogen bond networks. A pyrimidine ring was shown to be a valuable functional substitution for the hydroxy-keto intramolecular hydrogen bond in a salicylic acid derivative[216] (**3.10**, Figure 3.14) and a similar bioisosteric relationship (between **3.12** and **3.13**, Figure 3.14) was applied to the design of

tyrosine kinase inhibitors.[217] In light of these considerations, we decided to investigate the synthesis of D-ring fused heterocyclic derivatives of E1 (Figure 3.14).

Pyrazole-based inhibitors

Two precedents in the literature deal with heterocyclic fused systems as inhibitors of 17β-HSD. A series of 2,3- and 3,4-fused ring steroidal pyrazoles were shown to inhibit bacterial 17β-HSD type 3 activity via tight binding interactions.[218] The putative mechanism of inhibition involves a ternary complex inhibitor-enzyme-NAD^+ stabilised by hydrogen bonding between the pyrazole moiety and the imidazole group of a histidine.

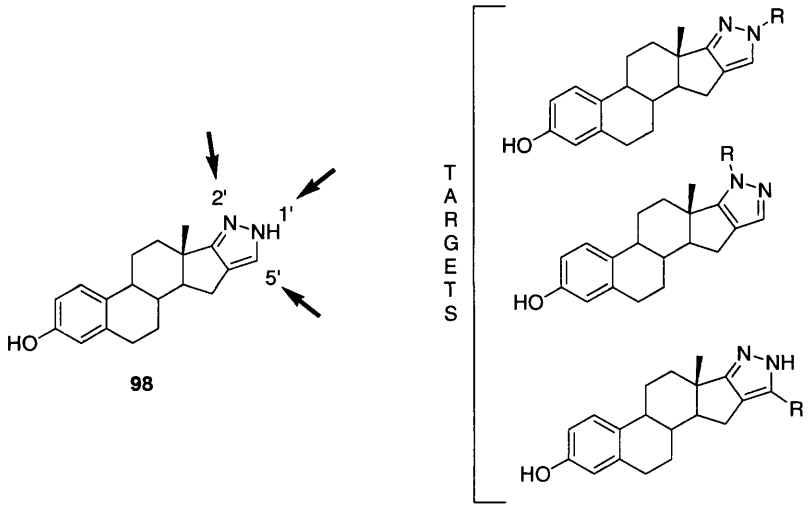


Figure 3.15 Sites of functionalisation of the pyrazole nucleus of **98** and future target compounds. (R = side-chain)

More recently, in an attempt to exploit potential pyrazole-histidine hydrogen bonding at the catalytic site, some D-ring fused pyrazole and isoxazole derivatives of E1 were prepared as inhibitors of 17β-HSD type 1.[219] Compound **98** (Figure 3.15) was shown to inhibit human placental 17β-HSD type 1 competitively with a Ki value of 4.1 μM which was better than the natural substrate used as an inhibitor (Ki = 9.5 μM). The 3-methoxy analogue of **98** was 3 times less potent than its parent, indicating the importance of the hydroxyl at C3 for affinity binding to the active site. This is

consistent with the fact that most steroidal inhibitors of 17β-HSD type 1 developed to date possess a free hydroxyl at C3. A model was proposed for the inhibitor-enzyme complex, where stabilisation occurs via specific hydrogen bonding between the phenol and the pyrazole groups of the steroid and histidyl residues of the active site.[219]

Although these studies were made obsolete by the later resolution of the three dimensional structure of human 17β-HSD type 1,[145] the pyrazole nucleus remains an attractive template for further investigation. With a basal inhibitory activity and a good affinity for the enzyme, it was anticipated that functionalisation of this moiety could result in an increased inhibition 17β-HSD type 1. It was therefore decided to investigate the SAR of a series of D-ring fused pyrazole analogues of E1. To this end, we investigated modifications of the heterocycle at the 1′, 2′ and 5′ positions in order to probe the space around the pyrazole nucleus in three directions (Figure 3.15).

Other heterocycle-based inhibitors

Unlike pyrazole-based derivatives of E1 (or other steroids), the biological activity of 6-membered fused heterocyclic steroidal derivatives is not well documented. Nevertheless, the interest in steroids bearing heterocycles fused to the A- or D-ring has led several groups to investigate the synthesis of steroidal pyrimidines with the heterocycle fused to the position 2,3 or 16,17.[220-225] We principally wanted to assess the potential activity against 17β-HSD type 1 of those derivatives where a nitrogen is incorporated as an isostere of the C17 carbonyl, and where the rest of the cyclic system mimics the putative hydrogen bond network described previously (cf. Figure 3.14) in **1**, **97** and possibly **64** and **71**.

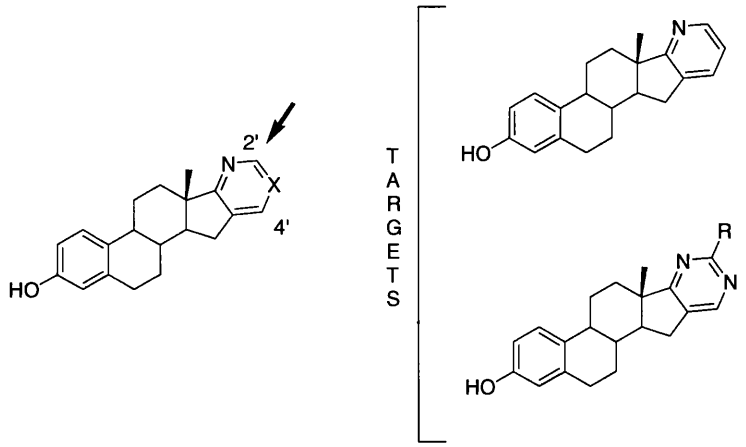


Figure 3.16 Target compounds for pyridine- and pyrimidine-fused heterocyclic derivatives of E1. The arrow indicates a potential site of functionalisation (R = side-chain)

Pyridine and pyrimidine heterocycles fused at the 16,17-position of E1 were therefore chosen as targets (Figure 3.16). Given that literature reports exclusively deal with C2'- and/or C4'-substituted pyrimidines (amino, alkoxy, thiacyanato) fused to the steroidal nucleus, a possible functionalisation at C2' was also envisaged.

3.2.2 Synthesis

i) N-alkylated pyrazoles

The synthesis of 1,2-azoles is generally performed via condensation of 1,3-diketones with hydrazine. In the case of E1 derivatives, early reports describe the preparation of 1,3,5(10)-estratrien-[17,16-c]-pyrazoles by reaction of hydrazine with 16-hydroxymethylene-estrone.[226-228] The latter can be obtained by condensation of E1 with ethyl formate.[229,230]

In order to access the desired *N*-alkyl derivatives, two pathways were initially envisaged starting from a protected 16-hydroxymethylene precursor (Figure 3.17): *(a)* formation of the pyrazole nucleus with hydrazine followed by *N*-alkylation (method A); *(b)* direct condensation of an alkyl hydrazine with a 16-hydroxymethylene derivative of E1 (method B). While method B represents a more

efficient approach to substituted pyrazoles, the *N*-alkylation reaction offers more flexibility with the greater range of alkyl halides commercially available, compared with fewer alkyl hydrazines.

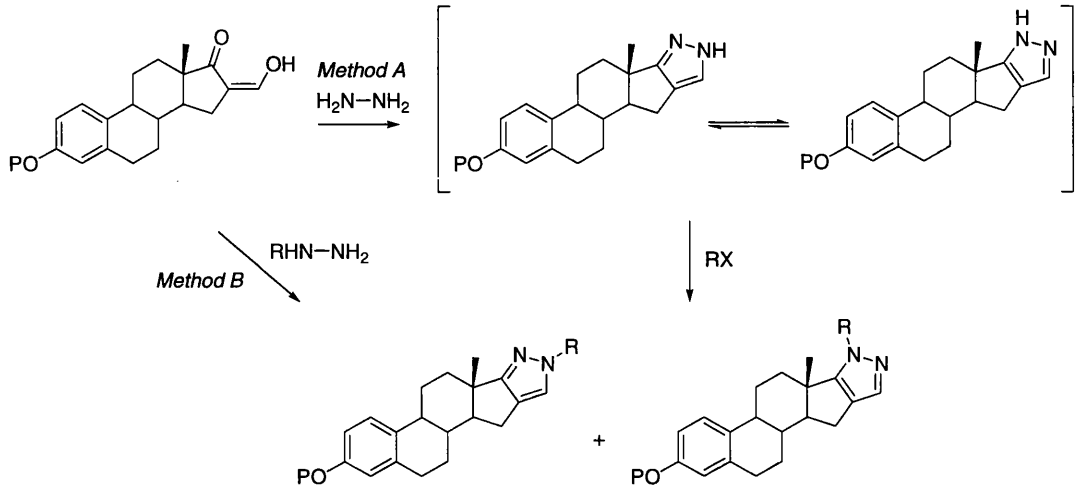
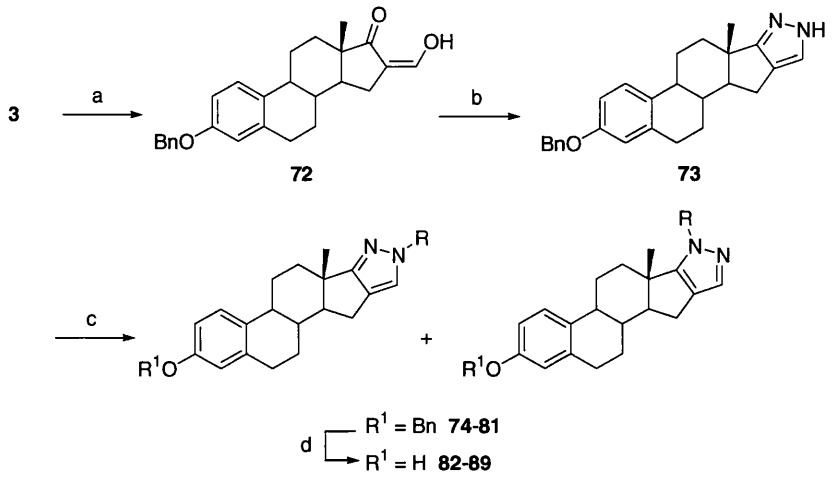


Figure 3.17 The two pathways to access *N*-alkylated pyrazoles. (P = protecting group, R = side-chain, RX = alkylating agent)

From a mechanistic point of view, it is anticipated that two regioisomers are formed during the *N*-alkylation reaction, in proportions reflecting either the predominance of one of the tautomers of the pyrazole (1′-*H* or 2′-*H*, Figure 3.17), or only the difference in sterical hindrance of the substituents introduced, possibly clashing with the C18 angular methyl group. If the regioisomers prove to be easily separable, two target compounds can be synthesised via this method.

Similarly, the reaction of unsymmetrical 1,3-diketones with alkylhydrazines generally gives a mixture of regioisomers, and their proportion depends on the nature of the substituents, as well as on the solvent and the reaction conditions.[231] However, in the particular case of E1 derivatives, the reaction is expected to commence at C1′ (the aldehyde carbon) due to the more reactive nature of the aldehyde vs. the C17 ketone, therefore only one product, the 2′-alkylated isomer may be formed using method B. In light of these considerations, method A was chosen to access the target compounds.



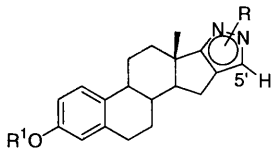
Scheme 3.4 Synthesis of *N*-alkyl D-ring fused pyrazole derivatives of E1. (a) $KOC(CH_3)_3$/toluene, HCO_2Et; (b) $H_2NNH_2.H_2O$, EtOH, reflux ; (c) NaH/DMF, RX ; (d) Pd/C, H_2, MeOH/THF.

Starting from 3-benzyl-*O*-estrone **3**, formylation at C16 was performed following an adaptation of the literature conditions (Scheme 3.4).[229,230] Using potassium *tert*-butoxide as a base, the resulting enolate of **3** was reacted with ethyl formate in toluene to give **72** in 86% yield. Subsequent condensation of **72** with hydrazine monohydrate in refluxing EtOH rapidly yielded **73** almost quantitatively. *N*-Alkylation was performed under standard conditions, using NaH in DMF, followed by treatment with the desired alkyl halide. As expected, each alkylation yielded two regioisomers, which were easily separated by flash chromatography in most cases.

We anticipated that for each alkylation reaction the same isomer would elute first from the chromatography column and decided to call the less polar compound (eluting first) 'regioisomer A' and the more polar (eluting second) 'regioisomer B'. Table 3.4 summarises the different isolated yields of regioisomers A and B for each reaction. As exemplified by the yields for the isomers **76/77** and **78/79**, the A:B ratio increases with steric hindrance. However, such an effect is not observed for **80/81** as total separation of the isomers by flash chromatography could not be achieved, thus the yields isolated do not reflect the proportions in which these compounds were formed. For the isomers **74/75**, the A:B ratio is close to 1, as predicted from the lower steric hindrance of a methyl substituent. Interestingly, a downfield shift of ca. 0.2 ppm was observed for the heterocyclic proton ($\delta_{5'\text{-H}}$, Table 3.4) in the regioisomer B. It is therefore envisaged that regioisomers can be differentiated on the basis of this

chemical shift difference, in addition to their difference in polarities. This may be particularly useful for the determination of the proportion of regioisomer A vs. regioisomer B formed during *N*-alkylation reactions, by examination of the ^{1}H NMR spectrum of the crude mixture. However, these observations did not allow to determine which alkylated product (1′ or 2′) corresponded to which regioisomer (A or B).

Table 3.4 Isolated yields and chemical shift of the heterocyclic proton (C5′-H) for regioisomers A and B in *N*-alkylated pyrazoles derivatives of E1. The substituent R is either at N1′ or N2′.

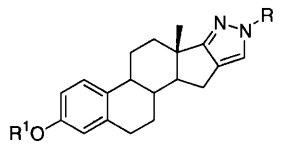


		regioisomer A			regioisomer B			
R	R^1	Compound	Yield (%)	$\delta_{C5'-H}$ (ppm)	Compound	Yield (%)	$\delta_{C5'-H}$ (ppm)	A:B
CH_3	Bn	**74**	33	6.97	**75**	35	7.15	0.94
$CH_2CH(CH_3)_2$	Bn	**76**	42	6.96-6.99[a]	**77**	19	7.17-7.21[a]	2.21
$CH_2CO_2CH_3$	Bn	**78**	48	7.07	**79**	20	7.25	2.40
$(CH_2)_2OCH_3$	Bn	**80**	27	7.09	**81**	23	7.19-7.21[a]	1.17
$(CH_2)_2CN$	TBDMS	**93**	8	7.10	**94**	12	7.25	0.67

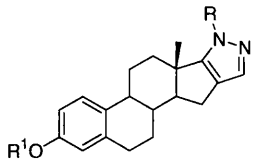
[a]Multiplet or under another signal

Subsequent deprotection of each regioisomer via hydrogenolysis gave the final products **82-89** in moderate to good yields after recrystallisation. A summary of the different *N*-alkylated pyrazole derivatives synthesised along with their numbering is presented in Table 3.5. Structural assignment for each regioisomer (1′- or 2-alkylated) is explained later.

Table 3.5 Numbering for the 1'- and 2'-alkylated pyrazole derivatives of E1 synthesised.



1'-alkylated

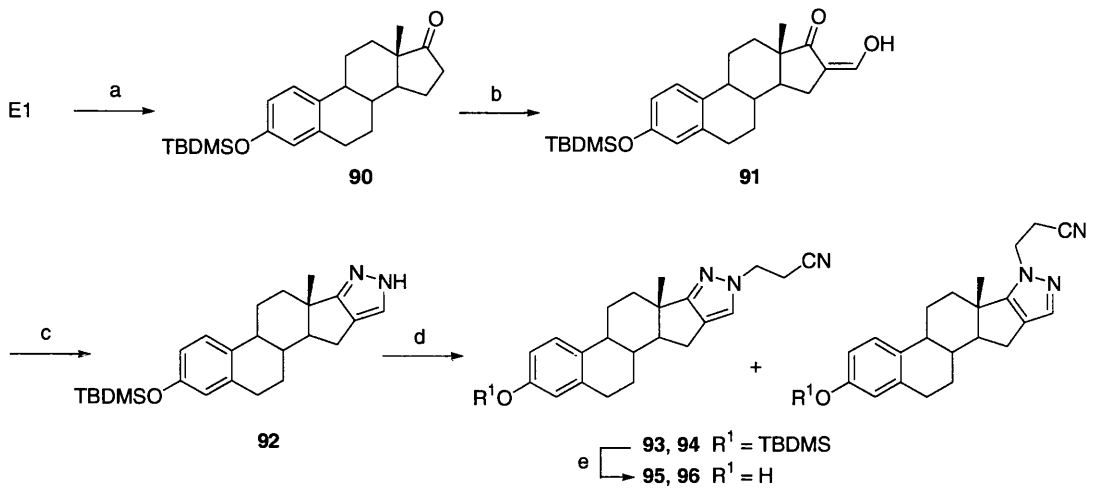


2'-alkylated

	R^1			R^1		
R	Bn	TBDMS	H	Bn	TBDMS	H
CH_3	**74**		**82**	**75**		**83**
$CH_2CH(CH_3)_2$	**76**		**84**	**77**		**85**
$CH_2CO_2CH_3$	**78**		**86**	**79**		**87**
$(CH_2)_2OCH_3$	**80**		**88**	**81**		**89**
$(CH_2)_2CN$		**93**	**95**		**94**	**96**

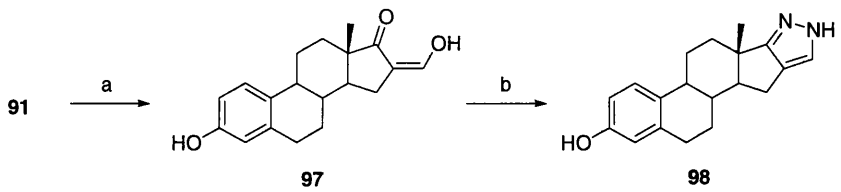
While the *N*-methyl, *N*-isobutyl, *N*-methylacetate and *N*-(2-methoxyethyl) pyrazole derivatives could be easily accessed following the reaction sequence in Scheme 3.4, the synthesis of the *N*-cyanoethyl analogue proved to be problematic. The use of bromopropionitrile to alkylate **73** only yielded mixtures in which the starting material was predominant. Conceivably, base-catalysed α-elimination on this halo-nitrile may have occurred, however this was not believed to be the major reason behind the lack of reactivity, as only a slight excess of base was used. On the other hand, a proton exchange between the deprotonated pyrazole and the alkylating agent may have promoted the formation of acrylonitrile, while regenerating the starting material.

These considerations prompted us to investigate the reaction of the pyrazole nucleus with acrylonitrile under basic conditions (Scheme 3.5). The expected reaction is a Michael-type 1,4-addition on the unsaturated system of the alkylating agent. For this pathway, the hydroxyl at C3 was protected with a TBDMS group since previous observations suggested that a cyanoalkyl moiety may be unstable under the hydrogenation conditions required for the cleavage of a benzyl protecting group.



Scheme 3.5 Synthesis of *N*-cyanoethyl derivatives. (a) Imidazole, TBDMSCl, DMF; (b) NaOMe/toluene, HCO_2Et; (c) $H_2NNH_2.H_2O$, EtOH, reflux; (d) $KOC(CH_3)_3$/toluene, CH_2CHCN; (e) TBAF/THF.

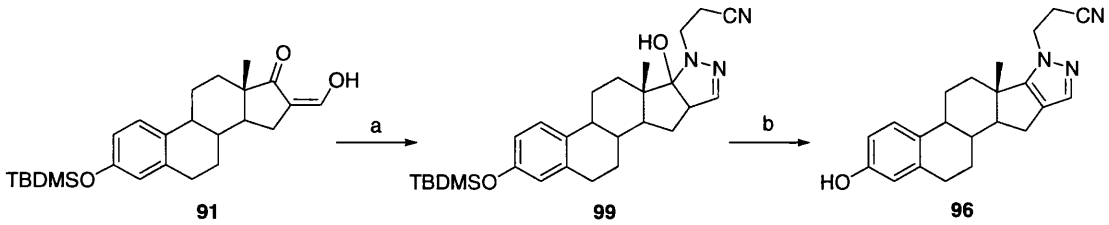
Protection of the 3-hydroxyl of E1 with a TBDMS group was achieved following a reported procedure, using imidazole and TBDMSCl in DMF.[232] The resulting compound **90** was obtained quantitatively and its formylation was performed following an analogous procedure to that described for the synthesis of **72** to afford **91** in 98% yield (crude material). Condensation of **91** with hydrazine monohydrate yielded the 16,17-fused pyrazole intermediate **92** with a yield of 81%, and subsequent reaction with acrylonitrile gave two regioisomers **93** and **94**. Unfortunately, the yields for this reaction were very poor and the separation of the products by flash chromatography was difficult, thus only small quantities of each regioisomer were isolated. Subsequent deprotection using TBAF in THF afforded the phenols **95** and **96**.



Scheme 3.6 Synthesis of 3-hydroxy-estra-1,3,5(10)-triene-[17,16-c]-pyrazole **98**. (a) TBAF/THF; (b) $H_2NNH_2.H_2O$, EtOH, reflux.

From the precursor **91**, it was also possible to access the deprotected and unsubstituted pyrazole derivative **98** which will be used as a reference compound for biological testing. Deprotection of **91** using TBAF in THF gave **97** in 69% yield, and subsequent annulation with hydrazine monohydrate afforded **98** with a yield of 64%.

Because of the poor yields for **93/94** following the procedure described in Scheme 3.5, we tried to access the corresponding final compounds **95/96** via Method B (see Figure 3.17 p128), which employs the condensation of a 16-hydroxymethylated precursor with an alkylhydrazine (Scheme 3.7). As discussed earlier, we expected the 2′-alkylated compound to be formed exclusively or at least predominantly.



Scheme 3.7 Synthesis of the *N*-cyanoethyl derivative **96** via Method B. (a) $H_2NNH(CH_2)_2CN$, EtOH; (b) TBAF/THF.

Compound **91** was therefore reacted with cyanoethylhydrazine in ethanol at room temperature and yielded a single product whose spectroscopic data did not match the structures of either **93** or **94**. The presence of an exchangeable proton at δ 5.97 and the absence of deshielded signals around δ 4.2-4.5 for the N-CH_2 moiety in the ^1H NMR spectrum of the product indicated that the condensation did not yield a fully aromatised heterocycle. Analysis by mass spectrometry showed an 18 mass unit difference between the mass of the product and that of **93** or **94**, suggesting that one molecule of water remained to be eliminated. The absence of a peak for the C17 carbonyl in the ^{13}C NMR spectra confirmed that cyclisation did occur. Assuming that the product obtained is a 2′-alkylated derivative, the presence of an inexchangeable, deshielded doublet at δ 6.74 in the ^1H NMR spectrum indicated that a double bond between C5′ and N1′ was formed. From these findings, the product was assigned the molecular structure of **99** which was formed in 73% yield, as a single diastereoisomer as evidenced by its ^1H and ^{13}C NMR spectra. Rapid conversion of the starting material into a single product was observed when monitoring the reaction

of **91** with cyanoethylhydrazine by TLC, suggesting that heating was not necessary. However, since partial condensation occurred to yield **99**, it is anticipated that heating the reagents in a refluxing solution of EtOH might promote water elimination and yield directly the fully aromatised product (**96**). Simultaneous deprotection and water elimination occurred when treating **99** with TBAF in THF overnight. The final product, obtained in 62% yield, was identical to isomer B (**96**) obtained in the synthesis described in Scheme 3.5.

Structural assignment of the regioisomers

Structural assignment was undertaken before deprotection of the precursors **74-81** and **93**, **94** as it was necessary to unambiguously determine which molecular structures corresponded to the regioisomers A and B. With the exception of **82/83**, the synthesised derivatives have not been reported previously and no NMR data were available for the *N*-methyl derivatives. On the basis of the yields reported in Table 3.4, we tentatively assumed that isomer A was the 1′-alkylated product since the A:B ratio was shown to increase with steric bulk. This was also consistent with the fact that deprotection of **99**, a 2′-alkylated compound, gave **96**, a regioisomer B.

These findings were further corroborated by ^{1}H NMR-NOE difference experiments for compound **74** and **75**. This particular technique allows the detection of groups which are close in space to each other. Irradiation at the resonance frequency of a proton (or group of protons) of interest produces a signal enhancement for the protons nearby. The signal enhancement can be seen on a difference spectrum. Irradiation of a sample of **74** (regioisomer A) at the resonance frequency of the C18-H_3 signal only yielded background noise (Figure 3.18 p 136). The same experiment over **75** (regioisomer B) yielded a significant peak at δ 3.81, corresponding to the enhancement of the signal of the *N*-methyl group. In both experiments, the angular methyl was also coupled in space with two protons (or group of protons) of the steroidal backbone around δ 1.7 and δ 2.3, asserting the reproducibility of the experiment between **74** and **75**. The NOE effect is only noticeable over short distances, generally 2-4 Å, and was therefore only expected to occur between the methyl groups in a 2′-alkylated compound. Using a model in the three dimensional

molecule viewer Chem3D, the average distance betweens these protons was found to be of 4.3 Å for isomer B, while it exceeded 6 Å in the 1′-alkylated isomer. The *N*-methyl pyrazole derivative **75** was therefore assigned the structure of a 2′-alkylated compound.

Structural assignment of the other members of the *N*-alkylated pyrazole series was made by analogy with **74** and **75**. Besides the fact that for each alkyl motif the regioisomers A and B could be differentiated by their polarities, the ^{1}H NMR shift for the heterocyclic proton ($\delta_{5'\text{-H}}$, Table 3.4 p130) was always around 0.2 ppm higher in the B regioisomer. This provided an easy way to rapidly ascertain the structure of these *N*-alkylated products. Contrary to what we expected, the position of the alkyl group on the pyrazole nucleus did not considerably influence the chemical shift of C18-H_3.

The final piece of evidence for assigning the structures of the regioisomers A and B was provided by the X-ray crystal structure of the *N*-isobutyl derivative **84**. This compound was obtained after deprotection of the regioisomer A isolated after *N*-alkylation of **73** with isobutyl bromide. The experiment was performed on a crystal (approximate dimensions 0.50×0.50×0.30 mm) obtained by recrystallisation from MeOH. As predicted from the NMR experiments on **74/75**, **84** was found to correspond to the 1′-alkylated product (Figure 3.19 p137). The ORTEX[173] plot shows all four steroidal rings with the additional heterocyclic system fused to the 16 and 17 positions. The presence of a molecule of MeOH in the asymmetric unit indicates that **84** co-crystallised with the solvent, to which it hydrogen-bonds via O1. The hydrogen atom H2 of each unit (**84**-MeOH) is hydrogen bonded to N2′ of the next unit generated by the x, y+1, z symmetry transformation.

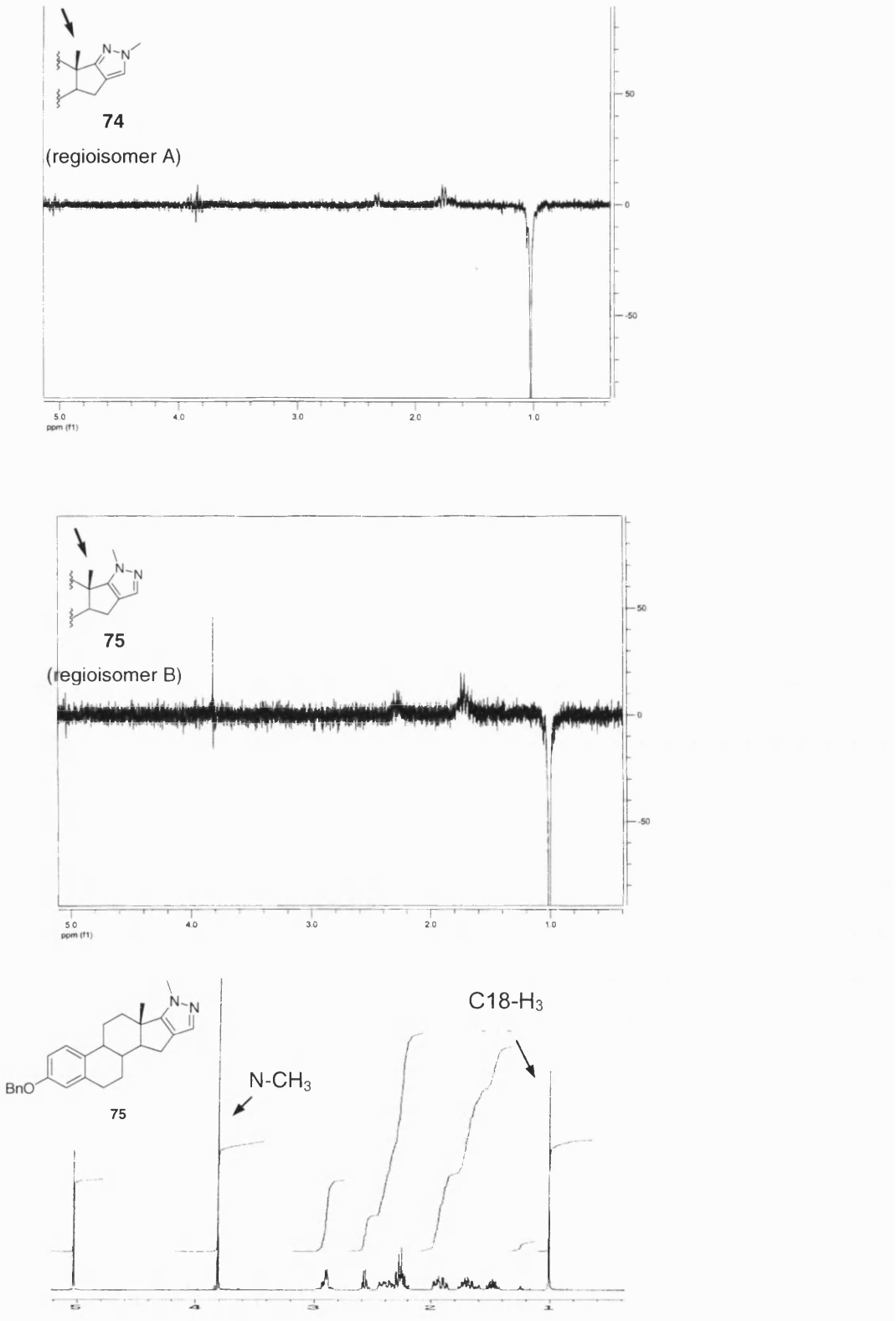


Figure 3.18 NOE difference spectra for **74** and **75**, and part of the ^{1}H NMR spectrum for **75** (400 MHz, $CDCl_3$). The bold arrows on the first two spectra indicate the signal whose frequency was chosen for irradiation (only the D-ring of the steroidal backbone is shown).

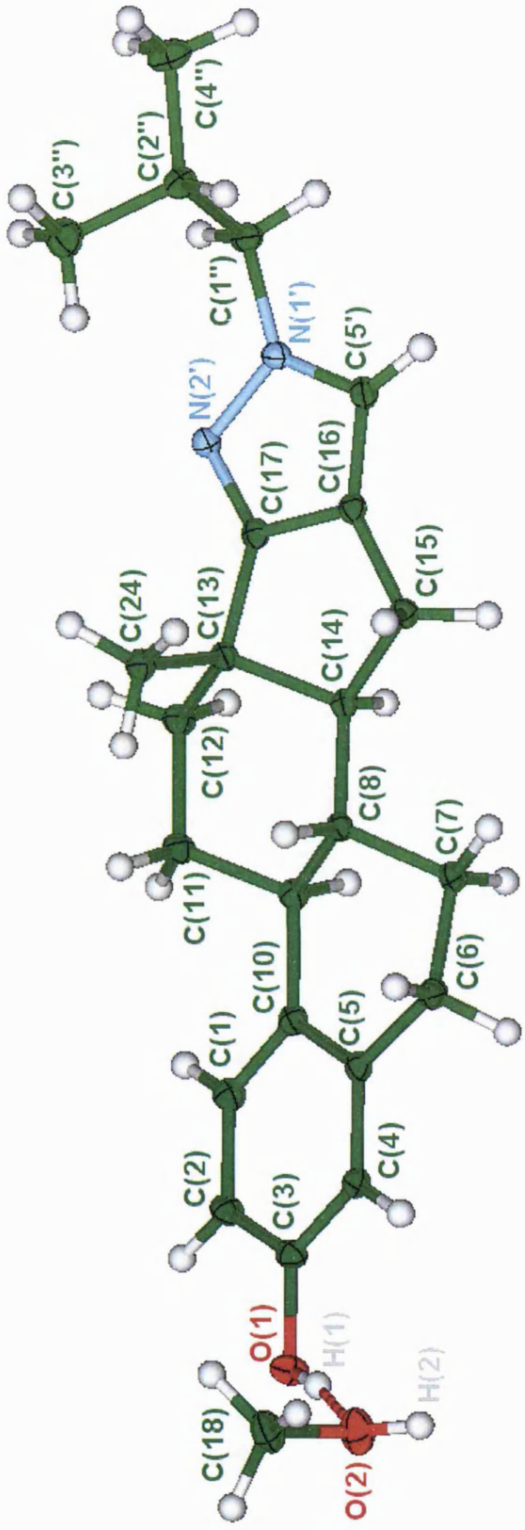


Figure 3.19 ORTEX[173] plot of the X-ray crystal structure of **84**. Ellipsoids are shown at the 30% probability level.

The bond lengths between the carbon atoms in the aromatic pyrazole system were 1.374 Å for C16-C5′ and 1.394 Å for C16-C17. The bonds between carbon and nitrogen atoms measured 1.359 Å and 1.334 Å for C5′-N1′ and C17-N2′ respectively, while N1′-N2′ measured 1.375 Å (for full details see Appendix 4). This is the first report of a crystal structure of a pyrazole fused D-ring derivative of E1 and this provides unambiguous proof of the molecular structure of these compounds and their regioisomers.

iii) C-alkylated pyrazoles

In order to fully explore the SAR around the pyrazole nucleus, we undertook the synthesis of C5′-substituted pyrazole derivatives of E1. Linkage with an alkyl group at C5′ can be achieved by condensing C1′-substituted 16-hydroxymethylene derivatives with hydrazine monohydrate. Presumably, these 16-hydroxymethylene compounds can exist in four different tautomeric forms as shown in Figure 3.20. Because of the established preference to locate double bonds in an exocyclic rather than endocyclic position relative to a five membered ring, the predominant enol form is believed to be B. For clarity, such compounds will be referred to as tautomer B and their enol-ketone ratio in solution will be discussed later.

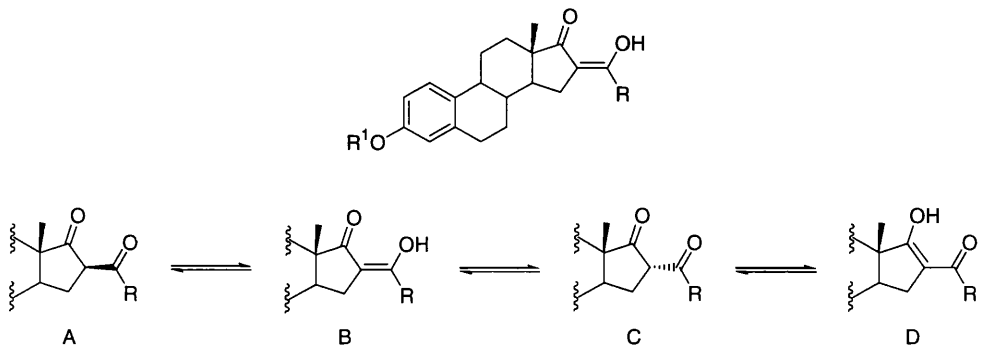
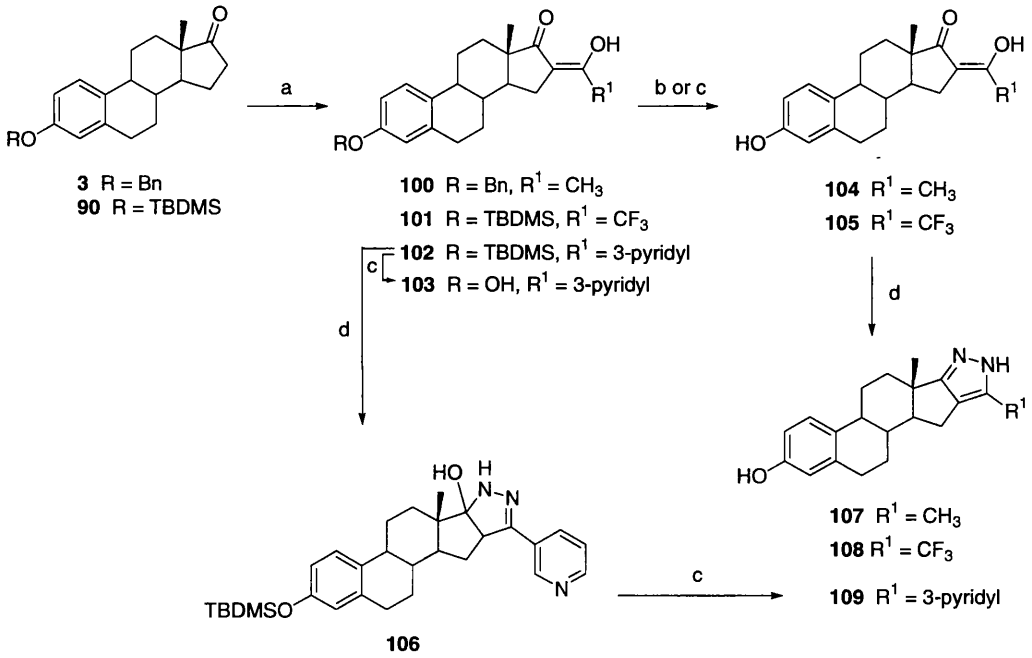


Figure 3.20 Tautomeric forms for 16-hydroxymethylene and 16-(1′-substituted)-hydroxymethylene derivatives of E1 (R = H or side-chain; only the D-ring of the steroidal backbone is shown).

Literature precedents for the synthesis of C1′-substituted 16-hydroxymethylene compounds include 16-acetylation[233] and 16-trifluoroacetylation[234] of 3-methoxy-estrone. Interestingly, not only can such compounds be used as synthetic

intermediates but their corresponding 3-hydroxy derivatives are analogues of **97** (16-hydroxymethylene-estrone) and therefore represent potential 17β-HSD type 1 inhibitors. The synthetic sequence envisaged includes the synthesis of C1′-substituted 16-hydroxymethylene intermediates, their deprotection and subsequent annulation into pyrazole derivatives (Scheme 3.8) to afford two series of compounds: 16-(1′-substituted)-hydroxymethylene and C5′-substituted pyrazole derivatives of E1. The hydrophobic methyl and trifluoromethyl moieties were chosen for preliminary SAR investigations. A 3-pyridyl group was also selected since high activity against 17β-HSD type 1 was observed for derivatives synthesised in our laboratories containing such a moiety around the D-ring.



Scheme 3.8 Synthesis of 5′-substituted pyrazole derivatives of E1. (a) $KOC(CH_3)_3$/toluene, R^1CO_2Et; (b) Pd/C, H_2, MeOH/THF; (c) TBAF/THF; (d) $H_2NNH_2.H_2O$, EtOH, reflux.

Following an adaptation of the procedure reported by Yoshioka *et al.*,[233] **3** was reacted with potassium *tert*-butoxide and ethyl acetate under refluxing conditions in toluene, to give **100** in 80% yield. Subsequent deprotection via hydrogenolysis afforded **104** which was then heated in a refluxing solution of EtOH in the presence of hydrazine monohydrate. The resulting product, the 5′-methyl pyrazole derivative **107** was obtained in a yield of 35%. An analogous sequence was applied for the synthesis of the 5′-trifluoromethyl derivative, starting from **90**. The 16-substituted

intermediate **101** was obtained quantitatively as a pure crude product from reaction of **90** with ethyl trifluoroacetate. The resulting product was deprotected with TBAF in THF to afford **105** in 60% yield. Reaction of the latter with hydrazine monohydrate, under the same conditions as for the synthesis of **107**, gave **108** in a yield of 60%.

Synthesis of the pyridinyl analogue was also carried out on the TBDMS-protected precursor **90**. Reaction of the latter with ethyl-nicotinate went to completion at room temperature affording **102** in a yield of 77%. Its deprotection gave **103** in 50% yield as a highly insoluble product, which prompted us to perform the subsequent condensation with hydrazine monohydrate on the protected precursor **102**. The product of this reaction was however not the expected aromatised heterocyclic derivative but the partially condensed product **106**, which was obtained in a yield of 89%, as a single diastereoisomer. The structure of this product was assigned after examination of its ^{1}H NMR spectra, where the signals for two exchangeable protons (OH and NH) could be seen at δ 5.97 and δ 7.26. Analysis by mass spectrometry also indicated an extra 18 mass unit for **106** compared to that of the expected aromatised product. As in the case of **99** (cf. Scheme 3.7 p133), deprotection using TBAF in THF afforded the deprotected and dehydrated product **109** in 75% yield.

Tautomerism in solution

As mentioned previously, the derivatives **100-102** and **103-105** may exist in several tautomeric forms and an enol-ketone equilibrium was seen for some derivatives in a solution of deuterated solvent, when collecting their ^{1}H NMR spectra. To the best of our knowledge, none of the 3-hydroxy derivatives **103-105** (or **107-109**) have been reported previously.

Compounds **100** and **104** were found to exist as a mixture of two tautomers in a solution of $CDCl_3$. While the protected compound **100** showed a high enol content vs. the ketone form (6:1), the derivative **104** displayed an equal proportion of each form. For the 3-methoxy derivative of **104**, Yoshioka *et al.*[233] reported a mixture of two enols in $CDCl_3$. In both **100** and **104**, however, we could clearly identify the peaks corresponding to C16-H, suggesting the presence of the ketonic and enolic tautomers (Figure 3.21).

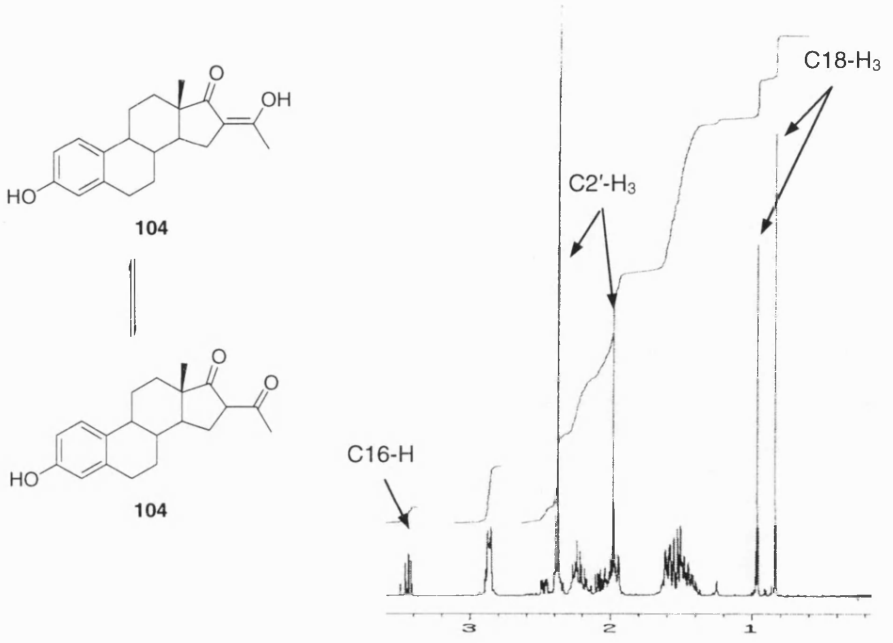


Figure 3.21 Part of the ^1H NMR spectrum of **104** in $CDCl_3$ (400 MHz).

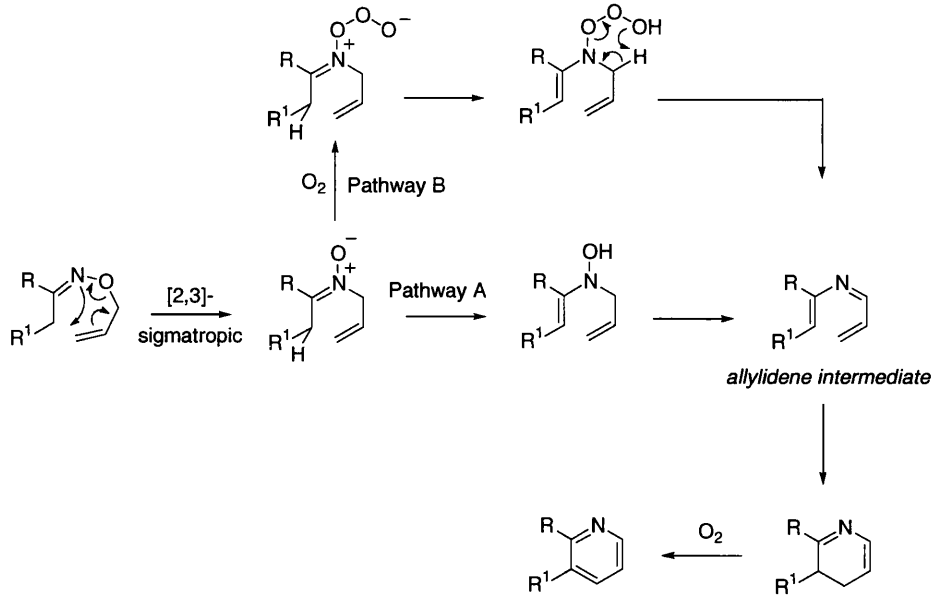
For the trifluoro derivatives **101** and **105**, the data of the ^1H NMR spectra were in accordance with the literature, where a 3-methoxy derivative of **105** was found to exist predominantly in the enol form.[234] As the enolic protons were not seen in our spectra (too broad), the structure of **101** was confirmed by ^{13}C NMR, where only one highly deshielded quaternary carbon signal was seen at δ 216.0. This peak was not coupled to the fluorine atoms of the trifluoro group, which confirmed the enolic character of **101**. If the structure had been ketonic (A or C, Figure 3.20) a signal corresponding to the second carbonyl at C1′ would have been expected, with coupling to the trifluoro group. It is believed that the electronegative character of the trifluoromethyl group, as well as the increased possibility for hydrogen bonding due

to the ability of fluorine atoms to bond OH, are involved in the stabilisation of the enolic form.[234,235]

Finally, the pyridyl derivatives **102** and **103** were also found to be totally enolised in $CDCl_3$. Collection of a spectrum for **103** was particularly difficult as this compound was sparingly soluble in $CDCl_3$. When the experiment was repeated in DMSO-d_6, the two enolic forms were seen as well as the ketonic form(s). Interestingly, when studying the ^1H NMR spectrum of the corresponding pyrazole derivative **109**, the two pyrazole tautomers could be seen. While one single C18-H_3 peak was observed, the peaks of the pyridyl protons were broadened with a shoulder effect. The signal for the NH moiety consisted of two singlets at δ 12.65 and δ 12.72, indicating that intramolecular hydrogen bonding possibly prevented free rotation of the pyridyl moiety around the C5′-C3″ bond, allowing as a result the observation of the two tautomeric forms of the pyrazole within the time of the acquisition of the ^1H NMR spectrum.

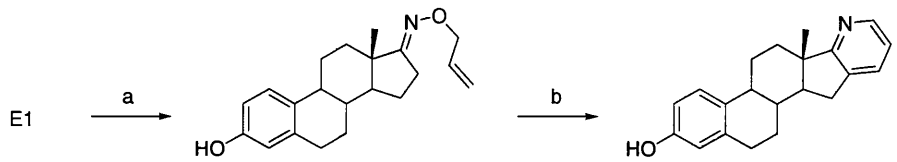
iii) Other heterocyclic derivatives

The synthesis of the [17,16-b]-pyridine derivative of E1 was envisaged via the thermal rearrangement of an *O*-allyl oxime precursor. We were prompted by the apparent extensive use of this method to generate pyridine heterocycles, although the yields reported for the corresponding reactions were generally moderate to low.[236-239] Recent approaches to annulation of a pyridine ring vary from one-pot[240,241] to microwave assisted procedures,[242] yet the development of a straightforward method to access such compounds still constitutes a synthetic challenge.



Scheme 3.9 Postulated mechanisms for the thermal rearrangement of *O*-allyl oximes into pyridines. Pathway A proposed by Kusumi *et al.*[236] and Pathway B proposed by Koyama *et al.*[237]

There is no clear report on the mechanism of the unusual thermolysis reaction of *O*-allyl oximes. It is thought to proceed via a multistep rearrangement, the first step being a [2,3]-sigmatropic rearrangement of the oxime to the corresponding nitrone (Scheme 3.9).[243,244] The investigation carried out by Koyama *et al.*[237] indicated that oxygen participated in the reaction in an ionic manner after nitrone formation (Pathway B), although this was not suggested in the earlier studies of Kusumi and coworkers (Pathway A).[236] In both cases, the nitrone is rearranged into an allylidene intermediate (Scheme 3.9), which then undergoes subsequent annulation and aromatisation.



110 **111**

Scheme 3.10 Synthesis of the [17,16-b]-pyridine derivative of E1. (a) $H_2NOCH_2CHCH_2.HCl$, NaOAc, $MeOH/H_2O$; (b) 230°C.

To the best of our knowledge, this reaction has never been applied to steroids, and it was anticipated that E1 could ideally be converted to a pyridine-fused derivative in

two steps. Following the adaptation of a literature procedure for the conversion of ketones to oximes,[155] the *O*-allyl oxime derivative **110** was easily obtained in 94% yield after treatment of E1 with *O*-allylhydroxylamine hydrochloride and sodium acetate in a mixture of MeOH and H_2O. The latter was then heated neat to 230°C for 46 hours to give a dark crude material from which **111** was isolated in spectroscopically pure form after repeated column chromatography. The harsh conditions required for this reaction, which resulted in loss of material through decomposition and a consequent tedious separation of **111** from the crude material might explain the low yield of 6% for this final product. It was envisaged that milder conditions would lead to better yields. An alternative would be to consider the annulation of a 1,5-diketone derivative of E1 (e.g. 16-propionaldehyde-estrone) in presence of ammonium acetate.[239]

The structure of **111** was assigned on the basis of spectroscopic evidences: a total of three additional aromatic signals were observed in the ^{1}H NMR spectrum of **111** around δ 7.0-8.4 which accounted for the aromatic protons of the pyridine nucleus (Figure 3.22); the absence of a peak for a C17 carbonyl above 200 ppm in the ^{13}C NMR spectrum of **110** and **111** was indicative of the conversion of C=O to C=N, with corresponding C=N peaks being observed in the δ 170 region. Mass spectrometry supported these structural assignments.

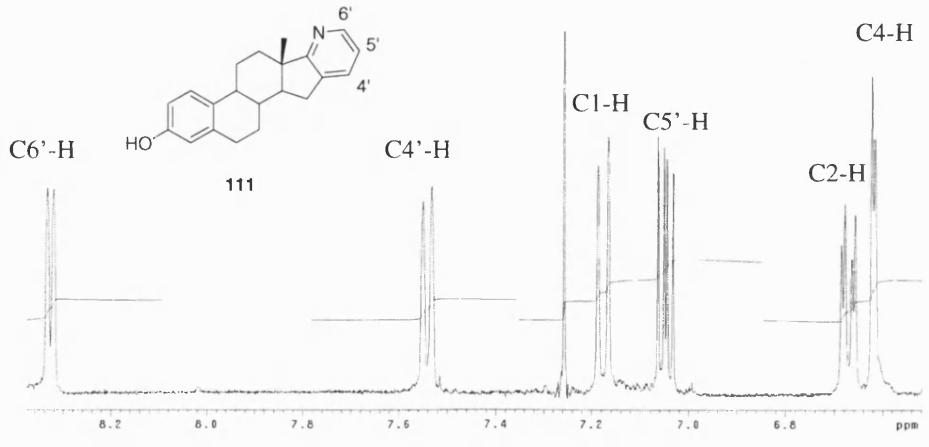


Figure 3.22 Aromatic region of the ^{1}H NMR spectrum of **111** ($CDCl_3$, 400 MHz).

However, attempts to access our next target, the [17,16-d]-pyrimidine derivative of E1 were met with little success. While examination of the literature provided several methods for the synthesis of steroidal fused pyrimidine derivatives substituted at C2' and C4',[220-225] no reports accounted for the annulation of an unsubstituted pyrimidine ring. Using analogous procedures to that employed for 1,3-diketone substrates,[245] we used the 16-hydroxymethylene derivative **72** as a starting material and attempted several condensation reactions without success. Treatment of **72** with formamidine hydrochloride in ethanol, in the presence of a base or in a pressure tube heated to 110°C only led to recovery of the starting material. Attempts to perform similar reactions with acetamidine hydrochloride or formamidine acetate also failed to yield the expected pyrimidine-fused product.

As an alternative to the study of steroidal fused-ring systems containing several nitrogen atoms, we envisaged the condensation of 16-hydroxymethylene derivative of E1 with several aminoazoles. Such reactions have been extensively studied at the A-ring of androgens by Bajwa and Sykes.[246-249] The authors also investigated these condensation with 3-methoxy-16-hydroxymethylene-estrone. It was expected that the corresponding 3-hydroxy azolopyrimidines derivatives of E1 would provide useful information on the extent of hydrophilic and pi-pi interactions at the active site of 17β-HSD type 1 and we therefore decided to undertake their synthesis. Three aminoazole substrates were chosen for this study: 5-aminotetrazole, 3-amino-1,2,4-triazole and 3-aminopyrazole (Figure 3.23).

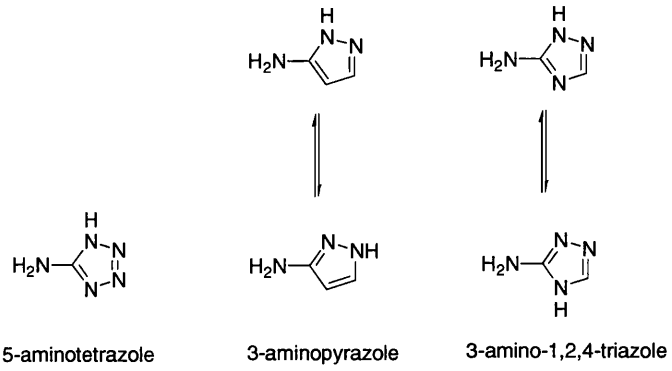


Figure 3.23 Structure and tautomeric forms of the aminoazoles substrates for condensation reactions.

Given that 16-hydroxymethylene derivatives of E1 are asymmetric β-diketones and that both 3-amino-1,2,4-triazole and 3-aminopyrazole can exist in tautomeric forms, we anticipated that a number of products may be formed as the result of each condensation reaction. The structure of the final product(s) depends on *(a)* the preferred site of attack of the primary amino group of the aminoazole to begin the reaction; *(b)* the nucleophilic nitrogen involved in the second condensation to form the final ring system. Depending on *(a)*, the resulting product has either a linear or angular structure (Figure 3.24).

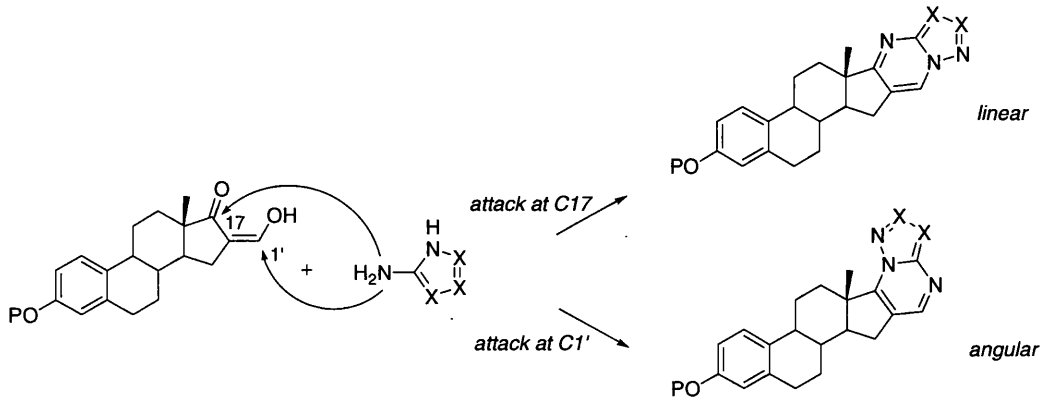
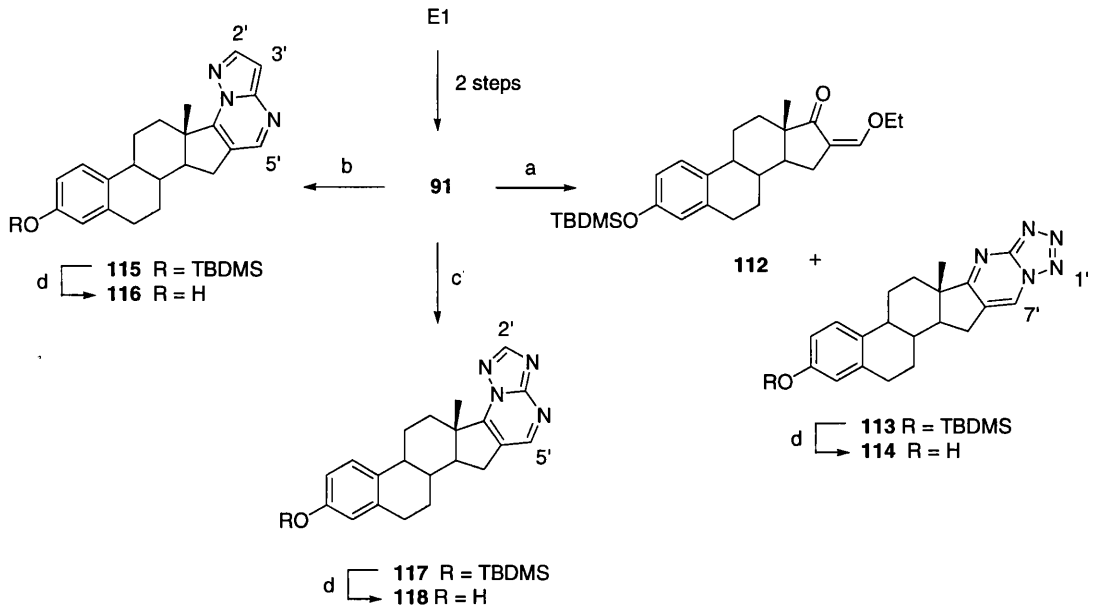


Figure 3.24 Condensation of a 16-hydroxymethylene derivative of E1 with an aminoazole. The linear structure results from a primary attack of NH_2 at C17, while the angular structure is formed after attack at C1′. (P = protecting group; X = CH or N).

As mentioned previously, a higher reactivity of the hydroxymethylene group vs. the carbonyl in 16-hydroxymethylene derivatives of E1 is expected to direct the condensation of the primary amino group towards C1′. This was corroborated by a literature report,[250] where the condensation of a 1,3-diketone with aminoazoles was proposed to yield angular products. In the case of 5-aminotetrazole, only one (angular) product was therefore expected to be formed.



Scheme 3.11 Synthesis of azolopyrimidine derivatives of E1. General conditions: aminoazole, EtOH, reflux. (a) 5-aminotetrazole; (b) 3-aminopyrazole; (c) 3-amino-1,2,4-triazole ; (d) TBAF/THF.

The condensation reactions were performed on the TBDMS protected precursor **91**, in a refluxing solution of ethanol in presence of a small excess of the aminoazole (Scheme 3.11). Upon reaction with 5-aminotetrazole hydrate, two products were unexpectedly formed. The heterocyclic fused derivative **113** was isolated in a yield of 32% along with a side-product which was identified as **112** (43% yield). The formation of **112** is believed to result from the action of 5-aminotetrazole as a base, which promoted a side reaction between the enol moiety of **91** and the solvent. Deprotection of **113** with TBAF in THF gave **114** in moderate yields. The latter was particularly difficult to purify as a result of its poor solubility in organic solvents.

Assignment of the molecular structure of **113** on the basis of its ^{1}H NMR spectrum alone faces the difficulty that the presence of only one additional aromatic signal at δ 8.74-8.75 provides little information about its spatial arrangement. Moreover, conflicting reports have been published on the condensation of 5-aminotetrazole with a β-diketone (such as 2-hydroxymethylene-cylcopentanone **3.14**, Figure 3.25). While Cook *et al.*[250] suggested that the product had an angular structure, Bajwa and Sykes[248] established with the aid of ^{1}H and ^{13}C NMR spectroscopy that the product was linear. In particular, it was established that in the ^{1}H NMR of the angular product

3.15, a sharp singlet was seen for C5-H, while the corresponding C8-H in **3.16** showed a small long range coupling with the protons at C7.

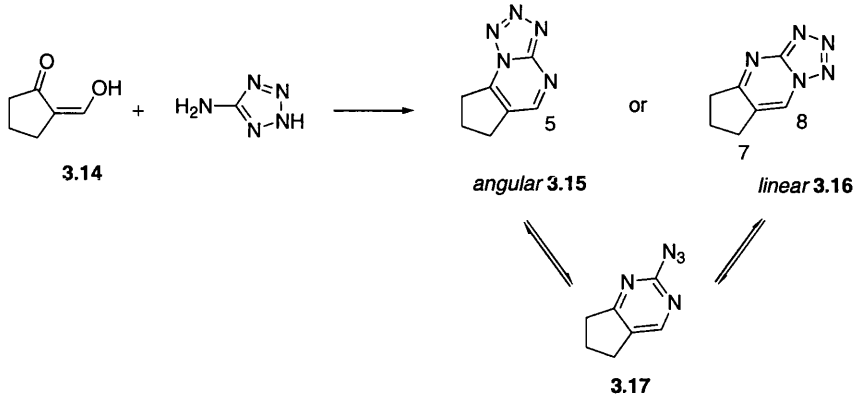
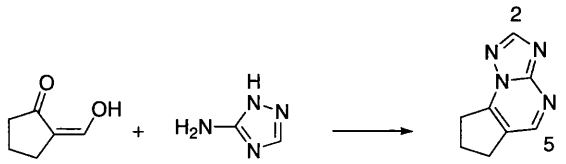


Figure 3.25 Reaction of 5-aminotetrazole with 2-hydroxymethylene-cylcopentanone **3.14** and putative products. The two products **3.15** and **3.16** may be interconverted via an azido-pyrimidine **3.17**.

Extensive structural studies on azolopyrimides by Bajwa and Sykes[246-249] however indicated that the condensation product of aminoazoles with **3.14** afforded mainly angular fused products. In the case of tetrazolo-pyrimidine derivatives, the angular structure may be formed from the linear initial product via an azido-pyrimidine intermediate (**3.17**, Figure 3.25).[248] Such investigations were extended and verified in steroidal substrates and the authors synthesised several D-ring fused azolopyrimidines derivatives of 3-methoxy-estrone. The structure of **113** was therefore assigned by analogy with the linear structure reported for the 3-methoxy analogue.[248] Interestingly, in the ^{1}H NMR spectrum of **113**, the signal for the heterocyclic proton (C7′-H) was a multiplet, which was in accordance with the linear fused product. This was not seen in ^{1}H NMR spectrum reported for the 3-methoxy derivative, possibly because of the lower resolution provided by the spectrometer used (100 MHz vs. 400 MHz in our case).

Condensation of **91** with 3-aminopyrazole gave **115** as the sole product of the reaction in 77% yield (Scheme 3.11). Although 3-aminopyrazole exists in two tautomeric forms, we anticipated the form protonated at N2 to react more readily with a β-diketone than the N1-protonated isomer, yielding the more favoured 6-

membered pyrimidine ring as opposed to a less stable 7-membered ring. Subsequent deprotection of **115** gave **116** in 91% yield. The structure of **115** was assigned by analogy with the 3-methoxy analogue reported in the literature.[249] In contrast to the condensation involving 5-aminotetrazole, the product obtained resulted from an angular fusion of the heterocyclic ring to the steroidal D-ring. The ^{1}H NMR spectrum of **115** (and **116**) showed characteristic doublets for C2′-H and C3′-H in the aromatic region while C5′-H was a singlet.



3.14 **3.18**

Figure 3.26 Reaction of 3-amino-1,2,4-triazole with 2-hydroxymethylene-cyclopentanone **3.14**.

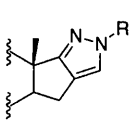
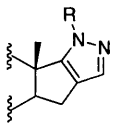
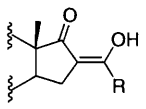
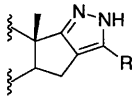
Condensation of 3-amino-1,2,4-triazole has not been reported on steroidal precursors. Taking into account that both tautomeric forms of this aminoazole might condense with the β-diketone functionality, four possible isomeric products were envisaged. However, reaction of 2-hydroxymethylene-cyclopentanone (**3.14**, Figure 3.26) with 3-amino-1,2,4-triazole was shown to yield exclusively one angular fused product (**3.18**, Figure 3.26).[247] Accordingly, when the reaction was carried out on **91**, one azolopyrimidine derivative **117** was isolated in a yield of 50%. It was deprotected to afford **118** in 66% yield. The structure of **117** was assigned by analogy with the data reported for the non steroidal analogues,[247] in the same way as Bajwa *et al.* assigned the structure of other steroidal fused azolopyrimidines from NMR data on non steroidal related analogues. The additional aromatic signals of the heterocyclic system were seen as singlets at δ 8.50 and δ 8.72 in the ^{1}H NMR spectrum, which was in agreement with the chemical shifts of C2-H and C5-H in **3.18** (Figure 3.26) respectively of δ 8.42 and δ 8.65. However, it is anticipated that further structural investigations need to be carried out to fully establish the proposed structure for **117** (and **118**).

3.2.3 Results

i) Inhibition of 17β-HSD type 1 in vitro

The compounds synthesised were tested for their ability to inhibit 17β-HSD type 1 activity in T-47D cells. As a control of selectivity, type 2 activity was also measured for each compound in MDA-MB-231 cells. Table 3.6 gives the percentage of inhibition achieved for a 10 μM concentration of the inhibitor. The data for the unsubstituted pyrazole derivative **98** are also included.

Table 3.6 Inhibition of 17β-HSD type 1 (and type 2) by pyrazole derivatives of E1 and **105**. Results are expressed as a percentage of inhibition at 10 μM (± S.D. triplicate).

		% inhibition 17β-HSD at 10 μM			% inhibition 17β-HSD at 10 μM	
R	Compound	type 1	type 2	Compound	type 1	type 2
H	**98**[a]	97.2 ± 0.4	32.1 ± 3.0			
CH_3	**82**	94.3 ± 1.3	24.2 ± 3.9	**83**	42.5 ± 8.3	32.3 ± 6.4
$CH_2CH(CH_3)_2$	**84**	76.9 ± 2.5	60.8 ± 3.2	**85**	70.2 ± 3.5	44.1 ± 5.7
$CH_2CO_2CH_3$	**86**	95.1 ± 0.4	18.6 ± 1.3	**87**	79.0 ± 1.1	29.8 ± 1.4
$(CH_2)_2OCH_3$	**88**	94.6 ± 0.8	47.9 ± 3.5	**89**	82.9 ± 1.1	55.2 ± 1.7
$(CH_2)_2CN$	**95**	95.1 ± 1.2	43.8 ± 3.9	**96**	nd	nd
						
CF_3	**105**	86.5 ± 0.3	- 1.5 ± 7.6	**108**	74.5 ± 0.9	35.7 ± 1.9

[a]May exist as a 1′-*H* or 2′-*H* tautomer

In the *N*-alkylated pyrazole series, all the 1′-substituted compounds significantly inhibited 17β-HSD type 1 activity. An inhibition of 94% or higher is observed at 10 μM for **82**, **86**, **88** and **95**. The isobutyl analogue **84** was less potent than the other derivatives with a 77% inhibition of 17β-HSD type 1 at 10 μM. This compound was also the least selective for the isotype 1, with nearly 61% inhibition of 17β-HSD type

2. The most selective compound for the 1′-alkylated series were **82** and **86**, while **88** and **95** inhibited 17β-HSD type 2 in excess of 40%. The unsubstituted pyrazole precursor **98** was highly potent at 10 μM (97% inhibition) which was in agreement with the literature.[219]

Interestingly, the activity of the 2′-alkylated derivatives was significantly lower than that of the corresponding 1′-alkylated compounds, with inhibitory activities of 83% or below at 10 μM. Positioning the same side-chain at N2′ was detrimental for the activity against 17β-HSD type 1, especially in the case of **83**. While its N1′ analogue **82** achieved a level of inhibition close to 95%, compound **83** inhibited a mere 42% of type 1 activity at 10 μM. However, for bulkier side-chains, the difference between 1′ and 2′-substitution was less marked. The selectivity for type 1 was also worse for 2′-alkylated compounds.

In the series of the 5′-alkylated pyrazole derivatives and their hydroxymethylene precursors, one motif has been tested so far. The trifluoromethyl derivatives **105** and **108** were moderately potent against 17β-HSD type 1 at 10 μM. Compound **105** was particularly selective for type 1, with a total lack of activity against 17β-HSD type 2.

Table 3.7 IC_{50} values for inhibition of 17β-HSD type 1 by several pyrazole derivatives of E1. Results are expressed as means of at least two determinations.

Compound	IC_{50} (μM)
98	0.18
82	2.75
86	0.92
88	0.53
95	0.73

The IC_{50} values were determined for those derivatives whose potency was sufficiently high at 10 μM, namely the 1′ derivatives **82**, **86**, **88** and **95** along with that of **98** (Table 3.7). Clearly, the substitutions on the pyrazole nucleus were detrimental to the inhibitory activity, since the unsubstituted pyrazole **98** remained

the most potent derivative, with an IC_{50} of 180 nM. Unexpectedly, a methyl side-chain provoked the highest drop of activity with **82** being 15 times less potent than **98**. The methoxyethyl analogue **88** was the best inhibitor of the *N*-alkylated series (IC_{50} = 530 nM), and was only 3 times less active than **98**.

ii) Estrogenicity in vitro

Although the compounds synthesised in the course of this study proved to be less potent than the precursor **98**, we were interested in evaluating their estrogenicity to compare the effects of a side-chain vs. no side-chain. For this study, the best inhibitor of the *N*-alkylated series **88**, and a compound bearing a small moiety (**82**) were chosen. Their estrogenicity was assessed in a luciferase reporter gene assay, and the results are given as a percentage of the maximum activity observed for E2, at four different concentrations (Figure 3.27).

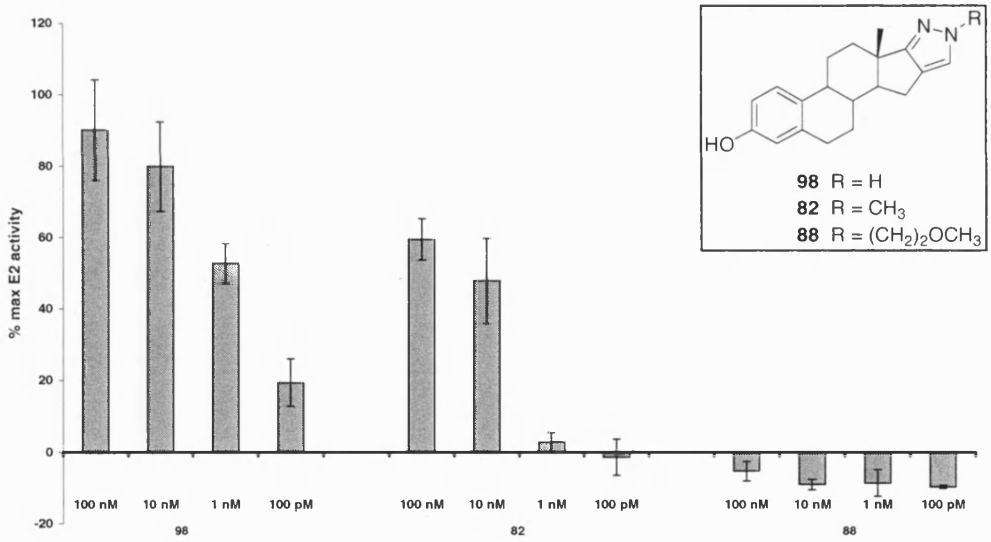


Figure 3.27 Estrogenic activity of **98**, **82** and **88** given as a percentage of E2's maximum activity.

From the data presented in Figure 3.27, it is clear that the side-chains contribute to a reduced estrogenicity of the pyrazole derivatives. While at 1 nM, the unsubstituted compound **98** is already half as estrogenic as E2, the estrogenic activity of derivatives **82** and **88** is less than 3% of that of E2 at the same concentration. In particular, **88** does not display any estrogenicity even at a concentration of 100 nM,

for which **82** and **98** are clearly estrogenic. Although **88** is 3 times less effective than **98** at inhibiting 17β-HSD type 1 activity, its estrogenic profile *in vitro* is highly encouraging, suggesting that it could be used to treat estrogen-dependent pathologies.

iii) Metabolic studies

Since it was anticipated that one or several of our inhibitors would be examined *in vivo*, a preliminary assessment of their biological profile was desirable. Metabolism mainly takes place in the liver, where cytochrome P450 catalysed-oxidation converts drugs to more polar conjugates for excretion. This however may take place before the drug has reached its site of action and elicited the desired response. A set of potential inhibitors was incubated with hepatic microsomes and their degradation monitored by HPLC (Table 3.8). The stability towards metabolism in the mouse and in the human hepatic systems was assessed and the half life ($t_{1/2}$) and hepatic clearance (Cl) for each compound were determined in both systems.

Table 3.8 Half life and hepatic clearance for the pyrazole derivatives **82**, **86**, **88** and **89** in the mouse and human. All incubations were carried out in duplicate.

	Mouse		Human	
Compound	$t_{1/2}$ (min)	Cl (mL/min/kg)	$t_{1/2}$ (min)	Cl (mL/min/kg)
82	< 30	> 23	< 30	> 23
86	< 30	> 23	< 30	> 23
88	> 60 (225)	< 11 (3.1)	39.9	17.4
89	45.6	15.2	> 60 (199)	<11 (3.5)

From these data, **82** and **86** appear to be metabolically unstable in both human and mouse, with both compounds having a half life below 30 minutes, and a corresponding hepatic clearance above 23 mL/min/kg. In contrast, the most promising alkyl-pyrazole inhibitor **88** was metabolically stable in the mouse and displayed moderate stability in the human system. Its regiosiomer **89** showed an even higher stability towards human microsomes, with a half life exceeding 60 minutes

and a hepatic clearance lower than 11 mL/min/kg. This compound was moderately stable in the mouse.

3.2.4 Discussion

i) Inhibition of 17β-HSD type 1 (and type 2) in vitro

The inhibition of 17β-HSD type 1 represents an important option in the search for endocrine agents to treat HDBC, yet this is a relatively recently explored area. There is a considerable need to identify lead structures and pharmacophores, and in an effort to develop new potent inhibitors of 17β-HSD type 1, we synthesised D-ring fused pyrazole derivatives of E1. Not only should the ideal 17β-HSD inhibitor be highly potent against its target, it also needs to be selective towards other 17β-HSD isotypes and devoid of estrogenic activity. All these requirements have resulted in only few candidates being identified, none of which has entered the clinic.

Preliminary work in our laboratory has identified the 16,17-fused pyrazole derivative of E1 **98** as a potentially useful template for the design of 17β-HSD type 1 inhibitors. This compound, which was previously shown to inhibit the enzyme with a Ki of 4.1 μM,[219] was functionalised at the 1′, 2′ and 5′ positions in order to build a comprehensive SAR. A series of 1′ and 2′ derivatives was synthesised simultaneously by *N*-alkylating the pyrazole nucleus. Small or bulky hydrophobic moieties and polar side-chains were introduced on the heterocycle with the aim to probe the active site for interactions that would enhance the affinity of the enzyme-receptor complex.

In vitro evaluation of the derivatives against 17β-HSD type 1 revealed a clear difference between the activity of the 1′- and 2′-substituted compounds. While the inhibitory activity of most 1′-alkylated pyrazoles was ca. 95% at 10 μM, the highest level of inhibition for 2′ derivatives was 83%. Surprisingly, the change of position of the substituents from N1′ to N2′ was the most detrimental to the activity for a small moiety, as exemplified by the 2′-methyl derivative **83**, which displayed a poor 42%

of inhibition of type 1 at 10 μM (vs. 94% for **82**). This clearly indicates that the active site has a limited tolerance for substituents pointing in the 2′ direction, where such moieties prevent the steroid from binding in a high affinity orientation. To some extent, this is in agreement with Poirier and coworkers' early findings that C16 derivatives of E2 are more potent inhibitors than their corresponding C17 analogues.[136] Unexpectedly, the drop in activity was less marked for the N2′ derivatives **85**, **87** and **89** (compared with **84**, **86** and **88**) indicating that a long flexible side-chain at N1′ or N2′ can be equally accommodated in the active site. The ability of such side-chains to interact in the active site via hydrophobic and/or polar interactions might also explain the higher activity of **85**, **87** and **89** compared with that of **83**. The methyl group in **83** might be too small to pick up hydrophobic interactions which could compensate with the steric clashes generated by this moiety at N2′.

In the 1′-substituted series, compounds bearing a polar side-chain exhibited the highest level of activity (~ 95% at 10 μM), with IC_{50}s ranging from 530 nM to 920 nM. The choice of side-chains bearing no more than 3 or 4 carbons was mainly driven by previous SAR[135,136] on E1/E2, where the active site showed a good tolerance for short moieties at the C16-position. A bulky hydrophobic group was however not well tolerated, as suggested by **84**, which was the weakest inhibitor of the series. Moreover, the *N*-methyl derivative **82** had a disappointing IC_{50} (2.75 μM) despite good inhibition of the enzyme at 10 μM, indicating that hydrophobic moieties placed at N1′ of a D-ring fused pyrazole nucleus do not contribute to specific binding/interactions with the active site.

The requirement for a polar moiety at N1′ to inhibit 17β-HSD type 1 might suggest that the side-chains of **84**, **86**, **88** and **95** interact with some residues of the cofactor binding domain. With the resolution of the three dimensional structure of 17β-HSD type 1, alone or complexed with substrate and/or cofactor,[145,146] it has been possible not only to identify the residues involved in high specificity binding of C18 steroids, but also those interacting with the cofactor. While several residues that interact with the substrate are hydrophobic (Val, Met, Leu, Pro, Phe), most of the residues that bind to the cofactor are polar (Cys, Ser, Arg, Asp, Asn, Lys). Such an observation

recently constituted the basis for the design of a series of 16β-propylaminoacyl derivatives of E2, where side-chains containing both hydrophobic and hydrophilic components were expected to interact with both the steroid and cofactor binding domains.[251] Unfortunately, the resulting compounds did not prove the validity of such an approach as they displayed very low inhibitory potency on 17β-HSD type 1 activity. In the case of the pyrazole derivatives **84**, **86**, **88** and **95**, it is conceivable that the pyrazole scaffold contributes to correctly orientate the polar moieties affording a good point of contact in the enzyme pocket.

Most of the pyrazole-based inhibitors showed selectivity for 17β-HSD type 1 inhibition. Poor selectivity was however observed for the derivatives **88** and **95** which inhibited isotype 2 activity by more than 40% at 10 μM. The isobutyl derivative **84**, which was the weakest inhibitor of 17β-HSD type 1 in the 1′-alkylated series, was the best type 2 inhibitor with more than 60% inhibition achieved at 10 μM. Since the three dimensional structure of 17β-HSD type 2 has not been resolved, it is difficult to assess which molecular features are likely to enhance the selectivity of a type 1 inhibitor towards type 2. From the activity observed for **84**, it is clear that hydrophobic interactions are important in the region of 17β-HSD type 2 active site corresponding to a N1′ substituent.

However, although most of the 1′-alkylated derivatives had IC_{50} values below 1 μM, it is clear that the alkylation of the pyrazole template has been detrimental to the activity. With an IC_{50} of 180 nM, the parent pyrazole **98** is more potent than any of its 1′- or 2′-alkylated analogues. The fact that **88** remains 3 times less potent than **98** suggests that the active site does not accommodate well the side-chains and/or a crucial interaction with the NH group is perturbed as a result of the alkylation. Both nitrogen atoms on the pyrazole nucleus may constitute a hydrogen bond donor (HBD) and acceptor (HBA) system, which interacts with the catalytic triad (Tyr155, Ser142 and Lys159) of 17β-HSD type 1 to result in a high affinity enzyme-inhibitor complex. Although polar interactions are favoured in this region of the active site, as demonstrated by the activity of **86**, **88** and **95**, they do not overcome the negative steric effect or compensate the loss of NH as HBD. We therefore hoped that

compounds of the 5′ series, where the HBA and HBD character of the heterocycle are conserved, would exhibit a higher level of activity. Unfortunately, too little data is available at present to draw a conclusion for such derivatives.

ii) Estrogenicity in vitro

Although **98** was reported to be a potent inhibitor of 17β-HSD type 1, there are no estrogenicity data available for this compound. In T-47D cells, this 16,17-fused pyrazole derivative of E1 was shown to inhibit 17β-HSD enzymatic activity with an IC_{50} of 180 nM and its 1′-methoxyethyl analogue **88** had an IC_{50} of 530 nM. Before undertaking further studies on compounds derived from the pyrazole template, it was necessary to assess their estrogenicity. Along with **98**, the estrogenic activity of **82** and **88** was evaluated.

Unexpectedly, **98** proved to be a full agonist of the ER, displaying estrogenic activity at concentrations as low as 100 pM. Although none of the alkylated derivatives were designed with the intention to decrease the potential estrogenicity of the template, we were pleased to see that the introduction of side-chains on the pyrazole nucleus considerably reduced its estrogenicity. Even a small moiety, such as a methyl group, introduced at the 1′-position dramatically decreased the agonist activity of the template. In particular, the analogue **88** was totally devoid of estrogenic activity over the range of concentrations tested, and therefore emerged as a strong candidate for further studies, being only 3 times less active than **98** against 17β-HSD type 1 activity.

Conceivably, the NH group of the heterocycle might be involved in the binding of **98** to the ER. In the same way as *N*-alkylation generates weaker inhibitors of 17β-HSD type 1 (vs. **98**), *N*-alkylation might result in compounds having a lower affinity for the ER. In addition, the *N*-methyl derivative **82** is estrogenic at concentrations above 10 nM, while **88** is not, suggesting that longer side-chain prevent binding to the ER. However, we cannot exclude that **88** might be an antagonist at the receptor. Such a possibility needs to be further investigated since a compound displaying both inhibition of 17β-HSD type 1 and antiestrogenic properties would be an ideal drug

for the treatment of HDBC. In this respect, Poirier and coworkers' attempts to design such a compound (i.e. dual action inhibitor) were not entirely successful. The derivatives that emerged from such investigations were moderately potent against 17β-HSD type 1 and not entirely antagonistic.[138]

iii) Metabolic studies

Given that alkyl substitution on a heterocyclic nitrogen is likely to give rise to metabolic instability due to dealkylation reactions and oxidation, some metabolic studies were undertaken on the *N*-alkyl pyrazole derivatives **82**, **86**, **88** and **89**.

Compounds **82** and **86** were unstable in presence of both human and mouse hepatic microsomes. The most likely metabolic pathway was *N*-demethylation for **82**, while the methyl acetate side-chain of **86** was expected to undergo rapid hydrolysis. To assess the influence of the location of the substituents of the heterocycle, the two regioisomers **88** and **89** were also assessed. While the 1′-alkylated derivative was moderately stable in the human system and stable in the mouse, the N2′ regioisomer had a greatly enhanced stability in the human system. This indicates that N2′ compounds are less likely to be metabolised *in vivo* than their N1′ analogues. Unfortunately, none of the 2′-alkylated derivatives was potent against 17β-HSD type 1. Nevertheless, this information is useful for the design of future inhibitors of the same series. So far, compound **88** seems to have several characteristics of a suitable candidate for *in vivo* studies: it is a potent inhibitor of 17β-HSD type 1 *in vitro*, with an IC_{50} of 530 nM in T-47D cells, totally devoid of estrogenicity *in vitro* and stable towards mice hepatic microsomes.

3.2.5 Conclusion

To address a growing need for agents able to block estrogen production in hormone-dependent cancer cells, we designed and synthesised a series of D-ring heterocyclic-fused derivatives of E1 as potential inhibitors of 17β-HSD type 1. In particular, a comprehensive SAR was built around the pyrazole nucleus fused at C16,C17.

Functionalisations were performed at the 1′, 2′ and 5′ positions, to probe the available space in three directions.

Efficient synthetic pathways were devised in order to access the target compounds in a minimum number of steps. In the pyrazole series, the 1′ and 2′-alkylated derivatives were isolated from the same reaction. While synthesising C5′-alkylated compounds, a series of C1′-substituted 16-hydroxymethylene derivatives of E1 could also be prepared.

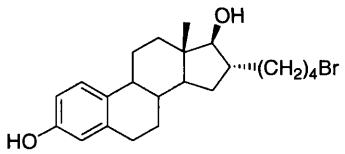
In vitro testing of the 1′ and 2′-alkylated analogues indicated that *(a)* N1′ was clearly the preferred position for the introduction of side-chains; *(b)* moieties with a polar character gave better inhibition of 17β-HSD type 1 than hydrophobic ones. It is suggested that substituents at N2′ contribute to negative steric effects which lower the activity of the corresponding derivatives while polar moieties at N1′ might interact with the cofactor binding domain. A methylacetate and a cyanoethyl derivative, **88** and **95**, were the most potent alkylated pyrazoles with respective IC_{50} values of 530 nM and 730 nM in T-47D cells. However, they were found to be less potent than their parent **98**, which had an IC_{50} of 180 nM in the same assay.

The introduction of a side-chain on the pyrazole nucleus did not yield better inhibitors of 17β-HSD type 1 than **98**, however this approach proved valuable when examining the estrogenicity data for some of the derivatives prepared. While **98** was a full agonist of the ER, the estrogenicity of its methyl **82** and methoxyethyl **88** analogues was comparatively low. The derivative **88** was also metabolically stable in mouse hepatic microsomes. Such results are highly encouraging as they indicate that *N*-alkylated derivatives of **98**, although less potent than their parent, might offer pharmacokinetic advantages and can be used in the treatment of hormone-dependent pathologies. However, before investigating further such compounds (e.g *in vivo*), it would be interesting to elucidate their mechanism of inhibition (tight binding, reversible) and whether a derivative such as **88** displays any antiestrogenic properties. The biological data for the C5′-alkylated compounds and the pyridine and pyrimidine-based heterocyclic derivatives should also bring further insight into understanding the molecular determinants responsible for17β-HSD type 1 inhibition.

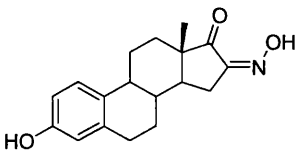
3.3 Small modifications on the estra-1,3,5(10)-triene nucleus

3.3.1 Rationale for novel targets

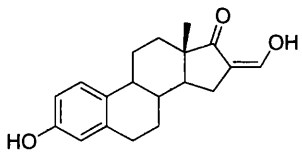
Since Poirier and coworkers' findings that 16α-bromobutyl-estradiol (**1.18**, Figure 3.28) was a potent inhibitor of 17β-HSD type 1,[135,136] most of the investigations dedicated to the search of agents blocking the activity of this enzyme have revolved around the D-ring, and in particular the 16 position of E1/E2. In a preliminary study aimed to identify pharmacophores or functionalities that could be exploited or optimised for the design of inhibitors, we evaluated the synthetic intermediates **1** and **97** (Figure 3.28) for their activity against 17β-HSD type 1. Both compounds emerged as potent inhibitors, with respective IC_{50}s of 1.10 and 0.11 μM in T-47D cells, which confirmed the validity of investigating the steroidal D-ring. As a result, part of our work was dedicated to the synthesis of pyrazole-based inhibitors of 17β-HSD type 1 (cf. section 3.2), where the D-ring fused heterocyclic system mimics a putative hydroxy-keto cyclic hydrogen bond network in **1** and **97**.



1.18



1



97

Figure 3.28 Structure of 17β-HSD type 1 inhibitors. Compounds **1** and **97** are intermediates synthesised in our laboratories.

In a concurrent approach, we wanted to identify another position of the steroidal backbone which would allow the introduction of functionalities designed to interact with residues of the active site. We therefore decided, prior to chemical synthesis, to examine the three dimensional crystal structure of 17β-HSD type 1 in complex with E2,[146] with a particular focus on the amino acids within close contact of the steroidal backbone. In order to narrow the area of space we intended to probe, several restrictions were applied regarding:

(a) the type of interaction desired between the target(s) amino acid(s) and the moiety to be introduced on the steroid. In general, an enzyme-inhibitor complex can be stabilised by electrostatic or hydrophobic interactions as well as hydrogen or covalent bonds. Although covalent bonds contribute to a higher stabilisation, hydrogen bonds or hydrophobic interactions are more easily achieved, and require the introduction of polar/hydrophilic or non polar/lipophilic moieties respectively on the inhibitor.

(b) the region of the steroid to target. We wanted to introduce a functionality at a position other than the D-ring which would not disrupt the high complementarity of the binding of the steroid to the active site. While E2 binds in a narrow hydrophobic tunnel, specific hydrogen bonds between residues of the active site and the hydroxyl functions at C3 and C17 contribute to stabilising the enzyme-substrate complex.[145,146,148] Structural modifications on the A-ring were therefore not envisaged, as any substitution in close proximity to C3 might be detrimental to hydrogen bonding at this position. We therefore focused on the steroidal B- and C-rings, for the introduction of moieties of small to medium size.

(c) the type of moiety to be introduced. In order to rapidly generate inhibitors, the structural modification envisaged had to be easily achieved. For the B- and C-rings, reports in the literature mostly deal with chemical synthesis of 6, 7 or 11 derivatives of E1/E2.

Therefore, our search was restricted to identifying potential residue of 17β-HSD type 1 that could interact via hydrophobic or hydrophilic interactions with a putative moiety or functional group situated at the 6, 7 or 11 position of E2.

The crystal structure of 17β-HSD type 1 in complex with E2 and $NADP^+$ was used for this study (code 1 FDT[146] in the Protein Data Bank[193]). From the examination of the amino acids present around the B and C ring, and in particular around the 6, 7 and 11 position, a serine residue in the vicinity of C6 clearly emerged as an attractive target. As shown in Figure 3.29, the distance between C6 in E2 and the β-hydroxyl moiety of Ser222 was found to be about 5 Å and it was anticipated that a small polar functionality introduced at the 6 position could hydrogen bond this hydrophilic amino acid.

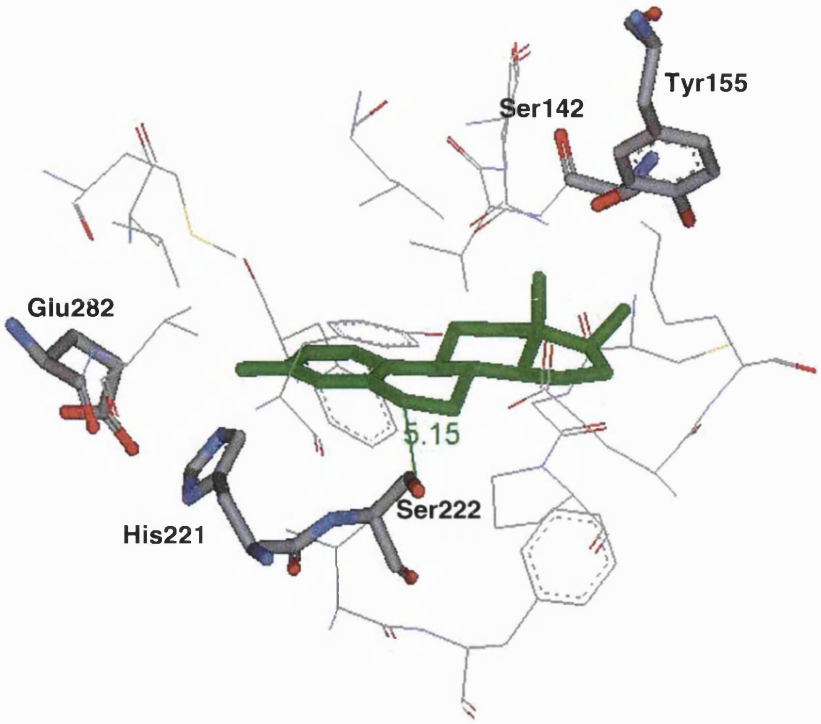


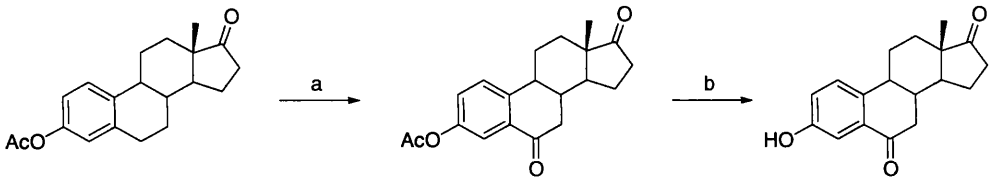
Figure 3.29 View of part of the active site of 17β-HSD type 1 in complex with E2 and $NADP^+$ ($NADP^+$ not shown). Critical residues and Ser222 are labelled as well as the distance between C6 in E2 and β-OH in Ser222.

Further to these observations, we decided to synthesise the keto derivative of E1 at C6, a known compound that can be accessed from E1 in a limited number of steps.[252-254] In addition, it seemed worthwhile investigating whether a moiety at C6 could potentially act synergistically with a functionality at C16 to enhance the inhibitory effect against 17β-HSD type 1, and 6,16- as well as 6,17-modified derivatives of E1 were also prepared. Estrogenic activity is undesired in endocrine therapy, therefore we chose to introduce modifications on the E1 template (3-hydroxy-estra-1,3,5(10)-triene-17-one) whose intrinsic estrogenicity is weaker than that of E2.

3.3.2 Synthesis

i) Preparation of 6-oxoestrone

The oxidation of the C6 benzylic position of E1 or E2 can be achieved using potassium permanganate or chromium (VI) derivatives.[252,253] However, low yields are reported for such reactions due to a number of side products being formed by cleavage of the B and C rings.[253] Few methods have been applied to steroids, especially to E1 or E2, and among recent procedures the use of *tert*-butyl hydroperoxide in the presence of chromium hexacarbonyl in refluxing acetonitrile was reported.[254] Although the yields for the oxidation of 3-methoxy-estrone were claimed to be higher than those obtained via previous methods, the difficulty of performing the reaction on a large scale prompted us to use an adaptation of Schwenk's procedure,[252] wherein E1 derivatives are oxidised in presence of chromic acid.



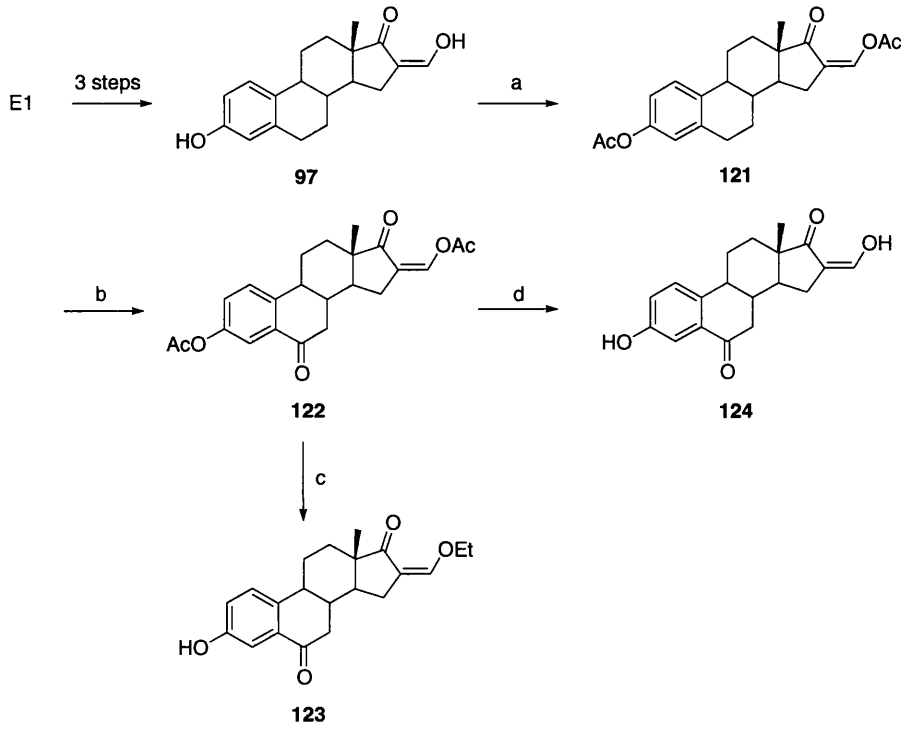
61 119 120

Scheme 3.12 Synthesis of 6-oxoestrone **120**. (a) CrO_3, $AcOH/H_2O$; (b) KOH/MeOH.

In order to prevent simultaneous oxidation at C3, the hydroxyl function of E1 was protected with an acetate group,[156] which was reported to be stable under oxidative conditions using chromic acid.[253] The resulting derivative **61** was then reacted with chromium trioxide in a mixture of acetic acid and water, below room temperature (ca. 15°C), so that the oxidation was relatively slow and mild. The oxidised product **119** was obtained in a yield of 20% after recrystallisation, which was consistent with previously reported yields.[254] Hydrolysis of the acetate group was then performed using KOH in MeOH to give **120** in 58% yield. The position of the carbonyl group in **120** was confirmed by the downfield shift of the C4-H signal in the ^1H NMR spectrum to δ 7.77 (vs. δ 6.81 in **61**), due to the deshielding of the benzylic carbonyl group.

ii) Combination of substituents at C6 and C16

In order to assess whether the putative effects of the ketone group at C6 would work in synergy with a moiety at C16 to inhibit 17β-HSD type 1, we decided to synthesise 6-oxo,16-hydroxymethylene-estrone (**124**, Scheme 3.13). Since the formylation of **120** (or a protected analogue) can take place at C7 or C16 due to the presence of two enolisable positions, the oxidation at C6 had to be performed after functionalisation at C16 (Scheme 3.13).



Scheme 3.13 Synthesis of 6,16-substituted E1 derivatives. (a) Ac_2O/pyridine, reflux; (b) CrO_3, $AcOH/H_2O$; (c) KOH/EtOH; (d) K_2CO_3/MeOH

Starting from E1, **97** was obtained in three steps following the sequence described previously (see section 3.2.2, overall yield 66%). The latter was then subjected to reaction with a large excess of acetic anhydride in refluxing pyridine in order to protect the derivative at C3. As predicted from the totally enolised character of **97**, C3-protection was accompanied by esterification of the enolic hydroxyl. The resulting bis-acetylated product **121** was isolated in a yield of 47%. As indicated by TLC ($CHCl_3$/EtOAc, 8:2) a second more polar product was also formed during the reaction. However, we were unable to isolate this product in a pure form after

purification of the crude material by flash chromatography due to the similarity between its R*f* (0.85) and that of **121** (R*f* = 0.91). Too little of this side-product was recovered to attempt further purification and examination of its ^{1}H NMR spectrum indicated the presence of two acetyl groups with corresponding methyl signals at δ 2.09 and δ 2.28 vs. δ 2.24 and δ 2.29 in **121**. Conceivably, a bis-acetylated derivative may also have been formed as a result of the esterification of the less stable enolic form of **97** (where the double bond is endocyclic).[255] Subsequent oxidation of **121**, using the same conditions as described for **61** (Scheme 3.12), gave **122** in a yield of 10%. The poor yield of this reaction may be attributed to the high number of side-products formed, due to the reactivity of the substrate.

Attempts to deprotect **122** using KOH in EtOH did not yield the expected product **124**, but a compound which was identified as **123**. Examination of its ^{1}H NMR spectrum indicated that both acetate groups were cleaved during the reaction. The presence of a triplet integrating for three protons at δ 1.35 and a deshielded signal for two protons around δ 4.1 suggested that an ethoxy function was added to the molecule. These findings were corroborated by mass spectrometry and it is suggested that **123** was formed as a result of a nucleophilic attack at C1′, occurring most likely on the protected enol, since AcO^- is a better leaving group than OH^-. Treatment of **122** under milder conditions (K_2CO_3 in MeOH) gave **124** in 54% yield. Mainly due to the low-yielding oxidation step (**121** to **122**), the final product **124** was obtained in only small quantities. Although the yields for this sequence can most likely be optimised, it would be of interest to investigate a more efficient and reliable method for the benzylic oxidation of E1 or E2.

iii) Combination of substituents at C6 and C17

To further explore the potential of functionalisation at the 6 position, we decided to convert the 6-keto group of **120** into another small moiety which would retain the hydrogen bonding properties of a carbonyl. An oxime group was particularly suitable for this purpose, given that it can easily be introduced in one step from a carbonyl compound without requiring any protection of the precursor. Moreover, the large

number of hydroxylamines available offers the possibility for further diversification of the template if required.

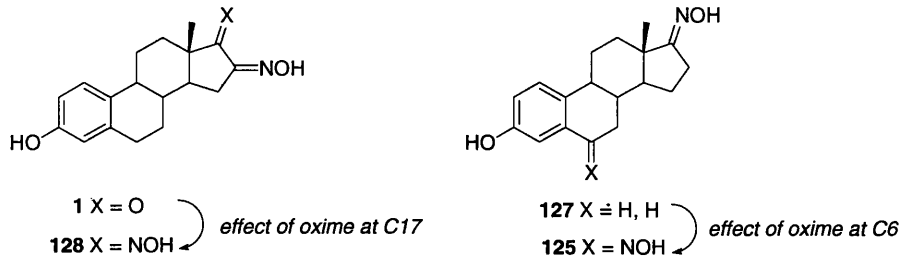


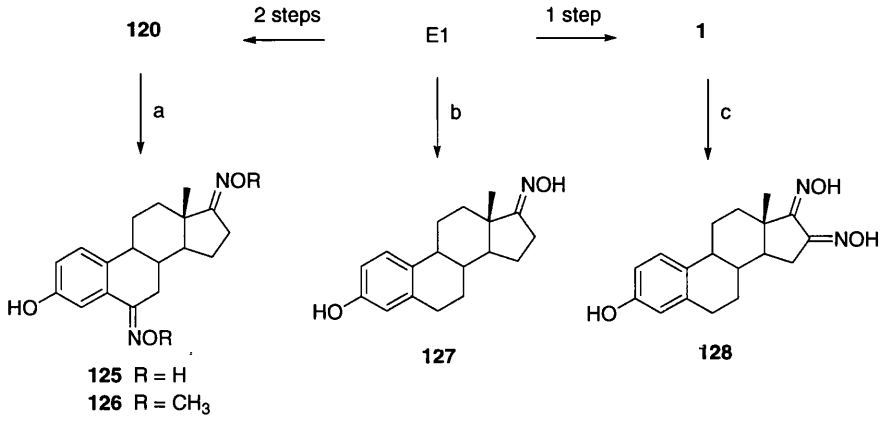
Figure 3.30 Structural modifications on the estra-1,3,5(10)-triene template and comparison of the effects of introducing an oxime at C17 and at C6.

Although we initially wanted to assess the effects of converting the carbonyl at C6 into an oxime, it was anticipated that starting from 6-oxoestrone, oximation would occur at both C6 and C17 carbonyl to yield **125** (Figure 3.30). Being unable to access selectively and in a short sequence the 6-oximino compound, two additional derivatives were prepared in order to gain information on the biological effects of adding and relocating oxime moieties around the estra-1,3,5(10)-triene ring system (Figure 3.30):

(a) **128**, a 16,17-bisoximino derivative, whose activity can be directly compared with that of **1** to assess the effects of replacing the carbonyl by an oxime at C17;

(b) **127**, a 17-oximino derivative which will allow evaluation of the effects of introducing an oxime at C6 by comparing its activity with that of **125**. It is anticipated that the activity of **127** can also be compared to that of the natural susbtrate for the enzyme, E1.

Both **127** and **128** were obtained after treatment of their respective precursors E1 and **1** with hydroxylamine hydrochloride and sodium acetate in a mixture of MeOH and H_2O (Scheme 3.14), following an adaptation of a literature procedure.[155]



Scheme 3.14 Synthesis of oxime derivatives of E1. (a) NHROH.HCl, NaOAc, MeOH/H_2O; (b) and (c) same conditions as in (a), with R = H.

Reaction of **120** with hydroxylamine hydrochloride or methoxylamine hydrochloride under the same conditions gave the analogues **125** and **126**. Compound **125** was highly polar and purification of the crude product by flash chromatography only afforded the product in 41% yield, while **126** was obtained with a yield of 92%. All these oxime derivatives existed as single geometrical isomers, as inferred from their ^{1}H NMR spectra. The derivative **127** is known to be formed as the *anti*-isomer, from the resolution of its crystal structure.[256] No further experiments were carried out on the synthesised derivatives to assess the configuration of the different oxime functionalities, although this could be worthwhile investigating if some of these compounds turn out to be potent inhibitors of 17β-HSD type 1.

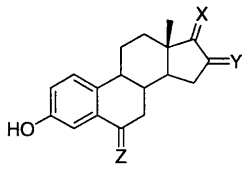
3.3.3 Results and discussion

i) Inhibition of 17β-HSD type 1 in vitro

The inhibitory activity of the different estra-1,3,5(10)-triene derivatives synthesised was assessed *in vitro*. Inhibition of 17β-HSD type 1 and 17β-HSD type 2 were examined in T-47D cells and MDA-MB-231 cells respectively. As in previous sections, the activity of the compounds against 17β-HSD type 2 was determined as a measure of selectivity of the inhibitors for the isotype 1. Table 3.9 gives the percentage of inhibition achieved for a 10 μM concentration of each compound as

well as the IC_{50} for some of the potent inhibitors. The data for E1, **1** and **97** were also included for comparison between structurally related analogues.

Table 3.9 Inhibition of 17β-HSD type 1 (and type 2) by estra-1,3,5(10)-triene derivatives. Results are expressed as percentage of inhibition at 10 μM (± S.D. triplicate). IC_{50} values are given as means of at least two determinations.



X	Y	Z	Compound	(% inhibition at 10 μM) 17β-HSD type 1	(% inhibition at 10 μM) 17β-HSD type 2	IC_{50} (μM)
O	H,H	H,H	E1	nd	nd	0.33
O	NOH	H,H	**1**	95.7 ± 1.0	5.0 ± 4.2	1.10
O	CHOH	H,H	**97**	96.6 ± 0.2	17.4 ± 1.9	0.11
O	H,H	O	**120**	98.4 ± 0.2	7.9 ± 10.7	0.34
O	CHOEt	O	**123**	84.7 ± 0.7	8.1 ± 6.4	nd
O	CHOH	O	**124**	97.3 ± 0.3	nd	0.70
NOH	H,H	NOH	**125**	83.1 ± 0.8	26.5 ± 0.5	1.90
NOMe	H,H	NOMe	**126**	31.3 ± 2.9	15.0 ± 4.3	nd
NOH	H,H	H,H	**127**	20.4 ± 6.5	nd	nd
NOH	NOH	H,H	**128**	54.9 ± 2.0	- 2.1 ± 4.9	nd

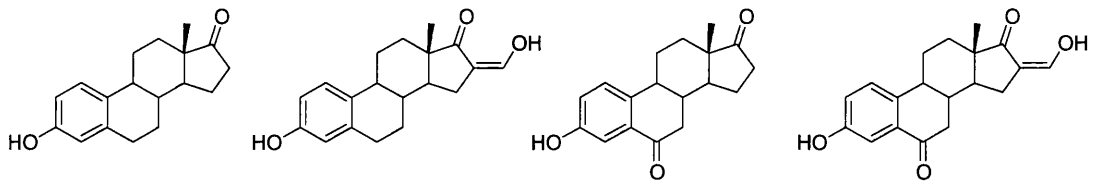
At 10 μM, most of the compounds tested displayed a high inhibitory activity against 17β-HSD type 1. In particular, the percentage of inhibition achieved by derivatives **120**, **123**, **124** and **125** was above 80% and therefore comparable to the reference compound **97**. Derivatives **120** and **124** were the most potent with ca. 97% of type 1 activity inhibited, and respective IC_{50}s of 340 nM and 700 nM. Although this indicates that targeting the 6-position alone or in combination with a functionality at C16 was not too detrimental to the activity, these derivatives are less potent than **97**. Compound **120** was equipotent to the natural substrate indicating that the 6-keto group was tolerated but did not enhance binding in the active site.

Overall, the oxime derivatives **125-128** were less potent than the other estra-1,3,5(10)-triene analogues synthesised in this study. In particular, they were less active than 16-oximinoestrone **1** which had an IC_{50} of 1.10 μM in T-47D cells. The best oxime analogue in this series was the 6,17-bisoximino derivative **125**, which achieved an 83% of inhibition of 17β-HSD type 1 at 10 μM. The presence of an oxime group at C6 and C17 did not abolish the activity of the template although with an IC_{50} of 1.90 μM, **125** was clearly less potent than **1**. The 16,17-bisoxime **128** had a weak inhibitory activity against 17β-HSD type 1 (55% inhibition at 10 μM), while **126** and **127** were practically inactive. Interestingly, introduction of an oxime at C17 in place of the carbonyl yielded the weakest inhibitor **127**.

Although type 2 activity was not determined for all the derivatives, a good selectivity was achieved for most of the analogues tested. The least selective compound was **125**, which inhibited the activity of type 2 by 26% at 10 μM.

Effect of a ketone at C6 and combination with a functionality at C16

From the activity data, it is apparent that the introduction of a ketone at C6 was not detrimental nor beneficial to the activity against 17β-HSD type 1 since **120** was found equipotent to E1. Nevertheless, the inhibitory activity of 6-oxoestrone **120** against 17β-HSD type 1 indicates that there is a potential small pocket in the active site to be exploited. As indicated by molecular modelling studies, polar groups at C6 might contribute to favourable interactions in the active site by hydrogen-bonding (as HBA) the hydroxyl group of Ser222 (a HBD). To some extent, the inhibitory activity of **120** confirms the predictive power of such studies.



E1
IC_{50} = 0.33 μM

97
IC_{50} = 0.11 μM

120
IC_{50} = 0.34 μM

124
IC_{50} = 0.70 μM

Figure 3.31 IC_{50} values for 6-, 16- and 6,16-modified compounds

Having identified C6 as a potential site of structural modifications, the next step was to determine whether the effect of a substituent at C6 and that of a moiety at C16 would be additive. To verify this possibility, the E1 derivative **124** (6-oxo,16-hydroxymethylene-estrone) was prepared. With an IC_{50} of 700 nM, **124** was found to be respectively 2-fold and 6-fold less potent than **120** (6-oxoestrone) and **97** (16-hydroxymethylene-estrone), suggesting that a synergistic or additive effect was not created by combining the two structural modifications. The drop in activity observed for **124** vs. **120** and **97** indicates that the ketone at C6 and the enol at C16 might be involved in mutually exclusive interactions in the active site. Conceivably, hydrogen bonding between Ser222 and the carbonyl at C6 may displace the inhibitor from the position adopted by **97** in the active site, resulting in the loss of the interaction between the side-chain of C16 and a putative hydrophilic residue. The enol moiety in **124** might also point towards a hydrophobic region of the enzyme. Conversely, an alignment of **124** which enhances interactions at the enol end might prevent hydrogen bonding between the C6 ketone and Ser222, or position the ketone in a hydrophobic environment.

Effect of an oxime at C6 and/or C17

We used the facile conversion of carbonyls to oximes to further investigate 6, 16 and 17 modifications on the estra-1,3,5(10)-triene nucleus. Given that the direct oximation of **120** yielded a 6,17-bisoximino derivative, we were unable to directly compare the effects of a carbonyl vs. an oxime moiety at C6, this requiring the selective oximation of **120** at C6. Nevertheless, the preparation of compounds **127** and **128** afforded some valuable insights into the potential of adding or relocating oxime functionalities around the estratriene ring system.

Conversion of the C17 carbonyl group into an oxime clearly reduced the activity of the corresponding derivatives. This was exemplified by **128**, a 16,17-bisoximino compound that was only half as active against 17β-HSD type 1 as its 16-oximino analogue **1**. Again, such a loss in activity might be explained by mutually exclusive interactions involving either oxime group in the active site.

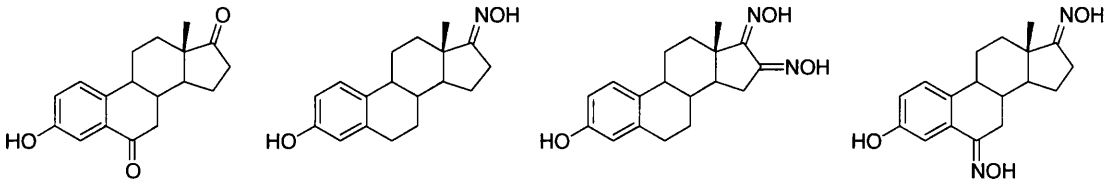


Figure 3.32 IC_{50} values for 6-, 17-, 16,17- and 6,17-modified compounds

However, the 17-oximino derivative **127** was found to be a poor inhibitor of 17β-HSD type 1 indicating that the replacement of the carbonyl at C17 by an oxime moiety was detrimental to the activity. As described by Ghosh *et al.*, the molecular basis for estrogen recognition involves highly specific interactions of the steroidal C3 hydroxyl and C17 carbonyl (or C17β-hydroxyl in E2) of E1 with residues of the active site.[148] While the hydroxyl at C3 is hydrogen bonded to His221 and possibly Glu282, the keto group at C17 interacts with Tyr155 and Ser142, which are part of the catalytic triad. It is therefore conceivable that the oxime moiety is unable to make specific interactions at the D-ring end and/or disrupts the triad, resulting in a less stable enzyme-inhibitor complex. This might be because of the bigger size of the oxime compared with that of a carbonyl group. Alternatively, if hydrogen bonding occurs between the oxime and residues of the active site, the steroid might be shifted to a position where interaction at the C3-end are no longer stabilising. The presence of an oxime functionality at C16 did not impart on the activity to the same extent as an oxime at C17, and the activity of **1** (vs. **127**) indicates that the carbonyl at C17 is crucial for binding while an oxime at C16 is tolerated.

Interestingly, **125** was found to be a better inhibitor of 17β-HSD than **127**, suggesting that the addition of an oxime functionality at C6, and the corresponding electronic interactions created in the active site, did overcome the inactivating effects of the oxime at C17. Similarly, when comparing the activity of **127** and **128**, it appears that the presence of an oxime at C16 in **128** contributes to a higher activity. When the oxime groups in **125** were replaced by *O*-methyl-oximes (cf. **126**), the activity dropped to 31% inhibition at 10 μM. This loss in activity may result from

steric clashes in restricted size pockets at C6 and/or C17, possibly combined with the HBA-only character of the moieties of **126** (vs HBA and HBD for the oximes in **125**).

Investigating the effects of introducing or relocating small isosteric functionalities on the estra-1,3,5(10)-triene ring system proved valuable as this contributes to a better understanding of the structural features that are involved in high affinity binding of a potential inhibitor to the active site of 17β-HSD type 1. At this stage, it is however not known whether the inhibitors designed act via a reversible or irreversible mechanism at the active site.

iii) Estrogenicity in vitro

To evaluate the potential of 6-, 16- and 17-modified estra-1,3,5(10)-triene inhibitors of 17β-HSD type 1 for their application as endocrine agents in hormone-dependent diseases, the estrogenic activity of some of the derivatives was assessed *in vitro*. Compounds **1**, **97**, **120** and **125** were tested in a luciferase reported gene assay and the results are presented in Figure 3.33. For each compound, the estrogenicity was measured at four concentrations (100 pM, 1 nM, 10 nM and 100 nM) and expressed as a level of luciferase activity. The luciferase level for background cells was also recorded and E2 was used as a reference in a 1 pM-1 nM concentration range. The derivatives **97**, **120** and **125** were tested side by side, while the activity of **1** was examined separately.

The 16-oximino derivative **1** was the most estrogenic compound of the series, being as estrogenic as 10 pM of E2 at a concentration of 100 pM. Its 16-hydroxymethylene analogue **97** was however less active at the ER, displaying pronounced agonist activity at a concentration of only 100 nM. Introduction of a ketone at C6 on the E1 template yielded **120** which was as estrogenic as E2 at a 100-fold lower concentration. Given that E1 was found to be about 100 times less estrogenic than E2 in our assays, the agonist activity of **120** was comparable to that of E1. Interestingly, **125** was devoid of estrogenic activity at concentrations up to 10 nM. Its level of

estrogenicity at 100 nM was comparable to that of 1 pM of E2, suggesting that **125** was ca. 10^5-fold less estrogenic than E2.

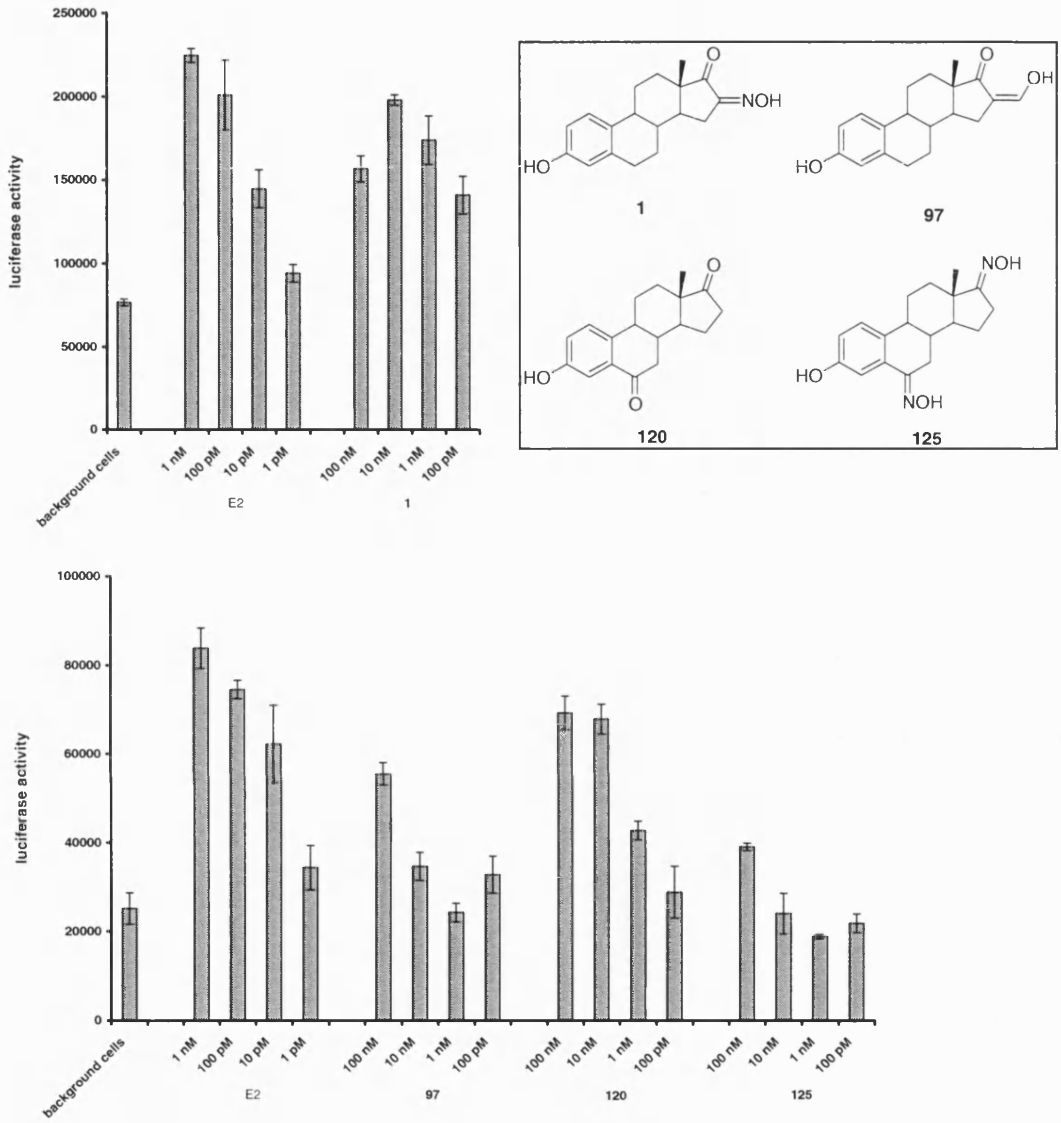
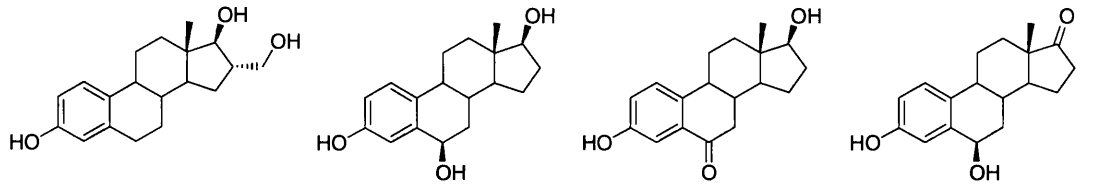


Figure 3.33 Estrogenicity *in vitro* for derivatives **1**, **97**, **120** and **125** tested at four concentrations. E2 was used as a reference. Results are expressed as mean ± S.D. of triplicate measurements.

All the compounds tested displayed some estrogenic activity, albeit at different concentrations and the strong disparity between the estrogenicity of estra-1,3,5(10)-triene derivatives bearing subtle structural modifications was noteworthy. For instance, **1** was found to be a potent agonist of the ER whereas **97** only showed a moderate level of estrogenic activity in the luciferase reporter gene assay. Given the

similarity in the structure of the two compounds such results are unexpected, in particular as both **1** and **97** bear small isosteric hydrophilic moieties at C16. However, in the same way as a potential different binding mode of **1** and **97** to the active site of 17β-HSD type 1 results in different levels of inhibition, both compounds may interact differently with the ER. This suggests that the oxime and the enol groups at C16 of E1 interact in a clearly distinctive way in biological systems, possibly because of the different electronic densities of the atoms in both moieties. A difference in the configuration of the hydroxyl groups relative to C=N and C=C bonds in the side-chains of **1** and **97** may also account for distinct affinities in the binding to the ER.



3.19 **3.20** **3.21** **3.22**

Figure 3.34 Structure of compounds previously investigated for their estrogenic activity.

Interestingly, the low estrogenicity of **97** may be correlated to the findings of Fevig *et al.* in their study on the ER binding tolerance of 16α-substituted derivatives of E2.[152] Although the ER had displayed a good tolerance and high affinity for small substituents at C16, the authors reported that highly polar derivatives, such as a 16α-hydroxymethyl analogue of E2 (**3.19**, Figure 3.34), did not bind to the ER efficiently. While the enol moiety of **97** does not strictly cover the same area of space compared with that of a 16α-hydroxymethyl group, these results suggest that there is a region of the ER where substituents of high polarity are not well tolerated. This was further corroborated by the recent study of Labaree *et al.*, which demonstrated that 16α-carboxylic acids derivatives of E2 were devoid of estrogenic activity as a result of a weak affinity for the ER.[257] More recently, the examination of the affinity of the four possible isomers of 16-hydroxymethyl-estradiol for the ER indicated that none of the compounds did bind the ER efficiently.[258] Yet, the reasons behind the estrogenicity of **1** are still unclear, and most likely the oxime moiety points towards an area of the

ER where hydrophilic interactions are favourable and contribute to stabilising the ligand-receptor complex.

While targeting the 6-position of E1 with small polar moieties was a potential approach for the design of potent inhibitors of 17β-HSD type 1, the estrogenicity of the potent derivative **120** was a major drawback to its further development as an endocrine agent to treat estrogen-dependent diseases. Oxygenated derivatives of E1 or E2 at C6 have been studied comparatively early, with 6β-hydroxyestradiol, 6-oxoestradiol and 6β-hydroxyestrone (**3.20**, **3.21** and **3.22**, Figure 3.34) being identified as metabolites of E2.[259] Although the activity of such compounds at the ER is well documented,[37,260,261] to the best of our knowledge, no data were available for **120**. In a study on some 40 derivatives of E2, Wiese *et al.* demonstrated that the introduction of a hydroxyl group at C6 (equatorial or axial) of E2 yielded compounds with weak affinity for the ER whereas 6-oxoestradiol displayed a 10-fold higher affinity than its hydroxylated analogues.[261] Clearly, a keto group placed at C6 of E1 (or E2) did not prevent high affinity binding to the ER, as exemplified by the estrogenicity of **120** at a concentration of 1 nM in our assay. Besides correlating our findings for **120**, these reported data[261] indicate that the estrogenicity of such compound could be abolished by reducing the carbonyl at C6 into a hydroxyl function. Whether this would be detrimental to the inhibitory activity of the derivative is unclear, although we expect the hydrogen bonding properties to be retained.

Interestingly, the conversion of both C6 and C17 carbonyl of **120** into oxime moieties (cf. **125**) did not produce an agonist of the ER. This was in agreement with a previous report where **127**, the 17-oximino analogue, was found to be about 40 times less estrogenic than E1 on oral administration.[262] These results confirm the requirement of a carbonyl (or hydroxyl) at C17 for high affinity binding to the ER and suggest that an oxime moiety does not mimic this group at the ER.

3.3.4 Conclusion

A small series of estra-1,3,5(10)-triene analogues was generated by the addition or relocation of ketone and oxime functionalities around the steroidal ring system. These compounds were designed and synthesised as potential inhibitors of 17β-HSD type 1. Further to investigations using the three dimensional crystal structure of the enzyme in complex with E2 and $NADP^+$, we primarily focused on introducing small modifications at the C6 position of E1. It was anticipated that a polar moiety at C6 would potentially interact with the hydrophilic residue Ser222, which was shown to come within close contact of the B-ring of E2, and in particular C6. In order to rapidly gain information on the structural features that would be crucial for high inhibitory activity, easy and convenient transformations were envisaged starting from E1 and most of the compounds were synthesised in a minimum number of steps.

Of the derivatives synthesised, 6-oxoestrone **120** was the most potent with an IC_{50} of 340 nM in T-47D cells for the inhibition of 17β-HSD type 1. However, no synergy was observed when the 6-keto group was combined with a functionality at C16 and 6-oxo,16-hydroxymethylene-estrone **124** had a disappointing IC_{50} of 700 nM. Mutually exclusive interactions between the moieties at C6 and C16 and the active site residues might explain the loss in activity in **124** compared with **120** and **97**. More importantly, *in vitro* estrogenicity studies revealed that **120** was an agonist of the ER, suggesting that some structural modifications should be envisaged before further investigating this template. Although these limited studies were not entirely successful in generating a potent inhibitor of 17β-HSD type 1, in particular **120** was 3-times less potent than **97**, the tolerance of the active site for a small polar moiety at C6 was demonstrated.

3.4 Potential dual inhibitors

3.4.1 Dual action agents: concept

A number of protein targets involved in estrogen production or action at the cellular level have now been identified. Besides agents that are designed to act as antagonists at the ER, inhibitors of steroidogenic enzymes represent a valuable alternative to treat hormone-dependent diseases such as HDBC by depleting estrogen levels.[72] In particular, the P450 aromatase, STS and 17β-HSD type 1 enzymes play a pivotal role in the biosynthesis of active estrogens by transforming inactive or androgenic precursors to E2 and Δ^5-diol, two potent agonists of the ER (Figure 3.35).

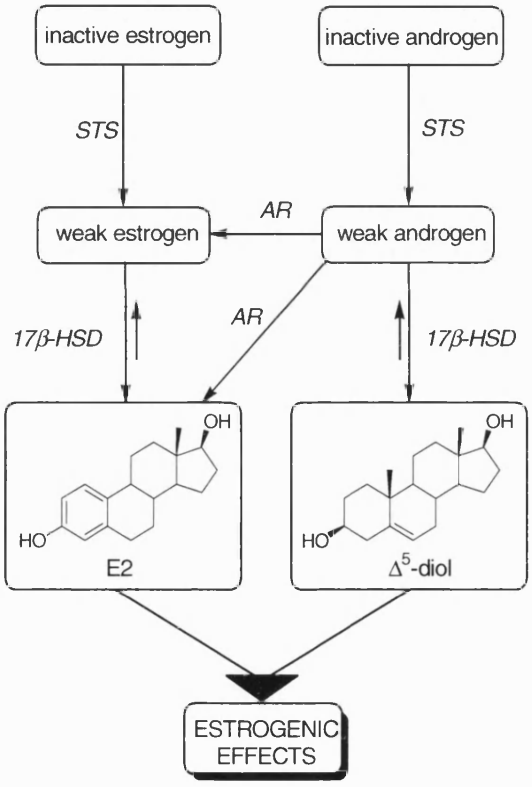
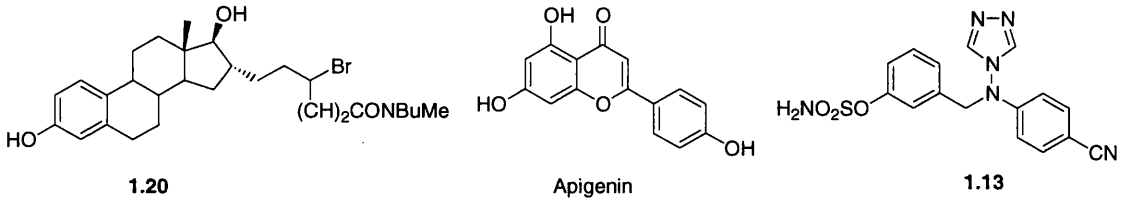


Figure 3.35 Schematic representation of the biosynthetic pathways leading to E2 and Δ^5-diol in normal and breast cancer cells (STS, steroid sulfatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; AR, aromatase).

While early investigations focused on blocking the conversion of androgens to estrogens via the inhibition of aromatase activity, it progressively became clear that

local production of estrogens from inactive precursors was an equally or even more important pathway.[73,78] The efforts therefore shifted towards the design of agents that could inhibit the estrogenic enzymes STS and 17β-HSD type 1, responsible for the activation of latent precursors to potent estrogens (Figure 3.35).

Most of the endocrine agents developed so far, or currently under investigation, solely target the ER or one of the steroidogenic enzymes in Figure 3.35. Yet, it can be conceived that a more effective reduction of estrogen levels could be achieved by the use of *(a)* a combination of two distinct inhibitors or *(b)* an inhibitor and an antagonist of the ER. However, previous experience of combining aromatase inhibitors with antiestrogens has not been convincing, partly due to pharmacological interactions between the drugs.[263] Dual action agents, which might be capable of providing the effects of a combination therapy as a single drug represent an attractive alternative.



1.20 Apigenin **1.13**

Figure 3.36 Structure of dual action agents: combined 17β-HSD type 1 inhibitor and antiestrogen (**1.20**), combined 17β-HSD type 1 inhibitor and aromatase inhibitor (Apigenin), combined STS and aromatase inhibitor (**1.13**).

There are few examples in the literature of dual action agents interfering with estrogen production or its action at the ER. In 1998, Tremblay *et al.* reported the synthesis and biological evaluation of 17β-HSD type 1 inhibitors that possessed antiestrogenic activity.[138] The best candidate (**1.20**, Figure 3.36) inhibited placental cytosolic 17β-HSD type 1 activity with an IC_{50} of 14 μM while being antiestrogenic at 1 μM. Several flavonoids were found to inhibit the activity of both 17β-HSD type 1 and aromatase enzymes, as exemplified by Apigenin (Figure 3.36) which inhibited by 78% and 95% respectively the activity of these enzymes in placental microsomes.[141,143] Recently, Woo *et al.* synthesised the first dual aromatase-STS

inhibitors (DASI) and validated their concept, with one compound (**1.13**, Figure 3.36) being potent against both enzymes *in vivo*.[111]

These findings prompted us to determine whether a similar concept could be applied to the dual inhibition of 17β-HSD type 1 and STS. Despite resulting in some potent inhibitors, our work and that of others failed to clearly identify a systematic functionality that would be the key to 17β-HSD type 1 inhibition. Nevertheless, most of the potent steroidal 17β-HSD type 1 inhibitors were shown to possess D-ring modifications in the form of a side-chain at C16 or more complex features such as a heterocycle fused at the 16,17-positions. In contrast, the pharmacophore for STS inhibition has long been identified as an aryl-*O*-sulfamate moiety[87] and it was anticipated that its introduction into a potent steroidal 17β-HSD type 1 inhibitor should confer some inhibitory activity against STS to the corresponding molecule, while possibly retaining the potency against 17β-HSD type 1 (Figure 3.37).

This approach seemed attractive for several reasons:
(a) the STS pharmacophore is easily introduced on a phenolic functionality, which all steroidal 17β-HSD type 1 inhibitors possess;
(b) the presence of a sulfamate at C3 may help decrease the intrinsic estrogenicity of the molecule;
(c) the phenolic precursor (i.e. the 17β-HSD type 1 inhibitor) may be released as a result of irreversible inhibition of STS via sulfamoylation of the enzyme resulting in an enhanced 17β-HSD type 1 inhibition;
(d) as shown on Figure 3.38, there is reasonable space in the active site of 17β-HSD type 1 to accommodate a sulfamate at the 3-position on an E1-based molecule.

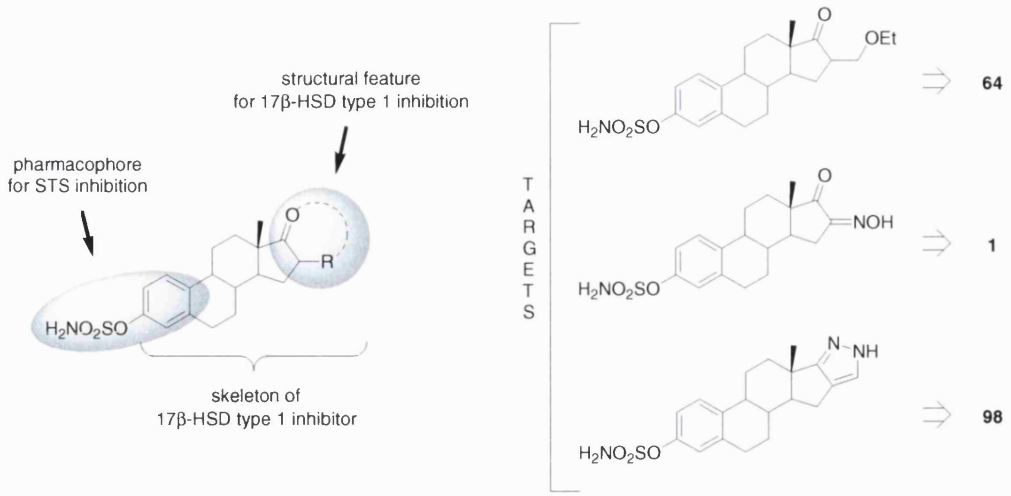


Figure 3.37 Approach to design of dual inhibitors of 17β-HSD type 1 and STS, structure of target compounds and corresponding precursors.

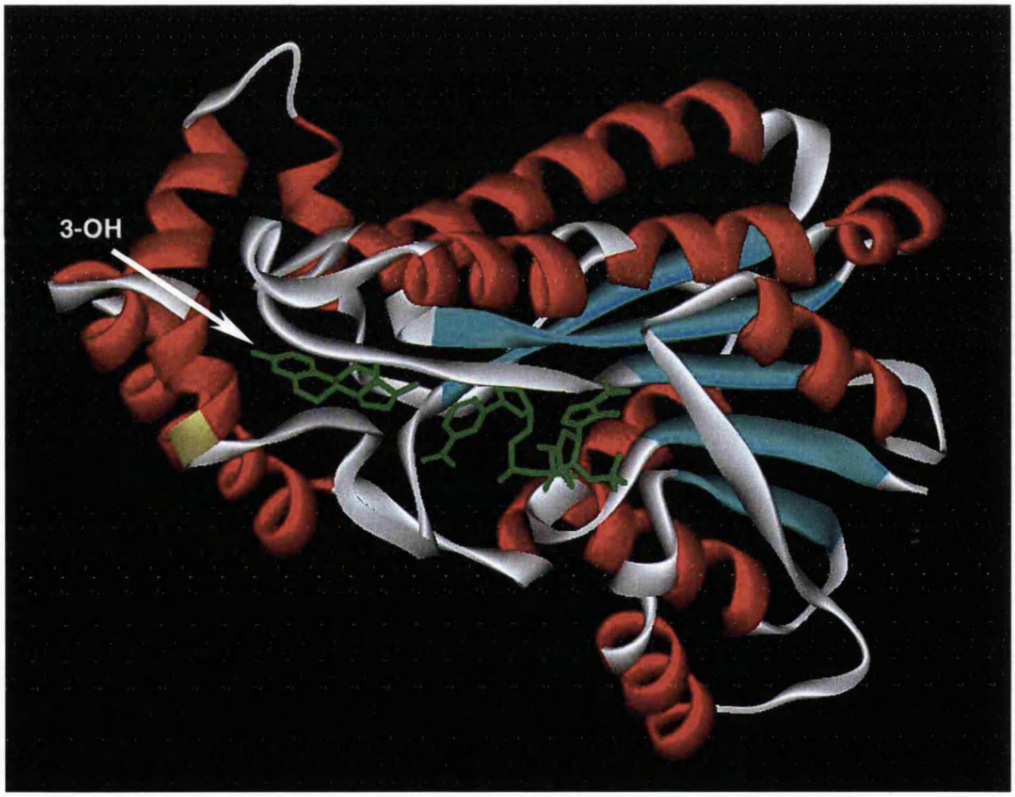


Figure 3.38 Three dimensional structure of 17β-HSD type 1 in complex with E2 and $NADP^+$, code 1FDT.[146]

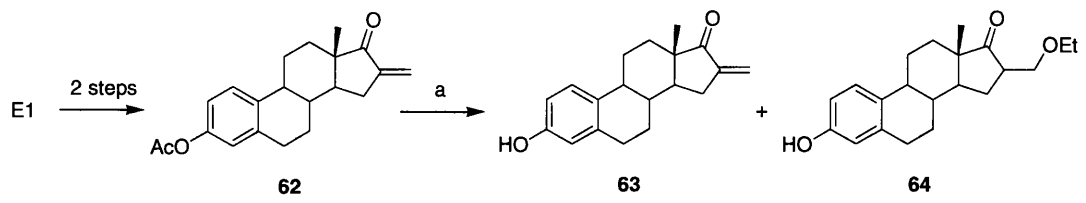
The 17β-HSD type 1 inhibitors **64**, **1** and **98**, with respective IC_{50}s of 320 nM, 1.10 μM and 180 nM in T-47D cells were chosen for this study. The structure of the corresponding sulfamoylated targets is presented in Figure 3.37. It was anticipated that the steroidal skeleton of these potential dual agents would ensure favourable hydrophobic interactions with the active site of both 17β-HSD type 1 and STS enzymes, while the A- and D-ring functionalities would provide additional affinities for the active site of STS and 17β-HSD type 1 respectively. However, it could not be ruled out that these pharmacophore manipulations might be detrimental to both activities, and although high activity is desired, a compromise situation where moderate potencies against the two enzymes are observed would be encouraging.

3.4.2 Synthesis

i) Preparation of the 16-ethoxyethyl derivative

Compound **64**, a side-product of the deprotection reaction of **62** (Scheme 3.15), emerged as a potent inhibitor of 17β-HSD type 1 at a time when we were investigating mechanism-based irreversible inhibition of this enzyme (see section 3.1). The high activity of this derivative might be attributed to the 16-ethoxymethyl side-chain that can interact via polar (cf. ether oxygen) and hydrophobic interactions in the active site of 17β-HSD type 1. In turn, this seems to indicate a certain potential for ether-type side-chains at C16 of E1 (or E2), and such a compound may also constitute the basis of new investigations. The moiety at C16 could be optimised by either modifying the length of the alkoxy group or that of the spacer between C16 and the oxygen atom of the ether functionality. However, for the time being, we were interested in exploiting the activity of **64** to design a dual inhibitor of 17β-HSD type 1 and STS.

As evidenced by ^{1}H and ^{13}C NMR spectroscopy, **64** was formed as a single diastereoisomer, however at this stage, the stereochemistry at C16 was unknown and to the best of our knowledge, this derivative (or a diastereoisomer) has not been synthesised previously.



Scheme 3.15 Synthesis of compound **64**, as a side-product of the deprotection reaction of **62**. (a) KOH/EtOH.

Before further investigations, the orientation of the ethoxymethyl side-chain at C16 was unambiguously determined by X-ray analysis of a crystal of **64** (approximate dimensions 0.40×0.13×0.04 mm) obtained from recrystallisation in EtOH. The ORTEX[173] plot in Figure 3.39 clearly shows the side-chain on the β-face of the steroid, with *R*-configuration at C16 (for full details see Appendix 3). To some extent, a β-stereochemistry may have been expected for the ethoxymethyl moiety of **64** since this product was formed as a result of the 1,4-addition of an ethoxide ion to the enone moiety of **62** (or **63**). In this reaction, the proton abstraction step occurs predominantly on the less sterically hindered face of the enone, i.e. away from the 18-methyl group.

Given that **64** was never obtained in yields higher than 10%, a new synthetic pathway was designed in order to provide sufficient quantities of this derivative for our present study and for possible future investigations. The aim was to stereospecifically introduce the ethoxymethyl side-chain with *R*-stereochemistry at C16. Examination of the literature confirmed that alkylation in the α position of the steroidal C17 ketone does not result in the desired stereochemistry since the C18 methyl group on the β-face directs the attack of any incoming electrophile towards the less hindered α-face.[135,136,138,232] Although Poirier *et al.* developed a methodology to access 16β-compounds stereoselectively,[135,138] we decided to exploit the directing effects of the angular methyl in a different way, namely through a hydrogenation reaction of an alkenyl group at C16.

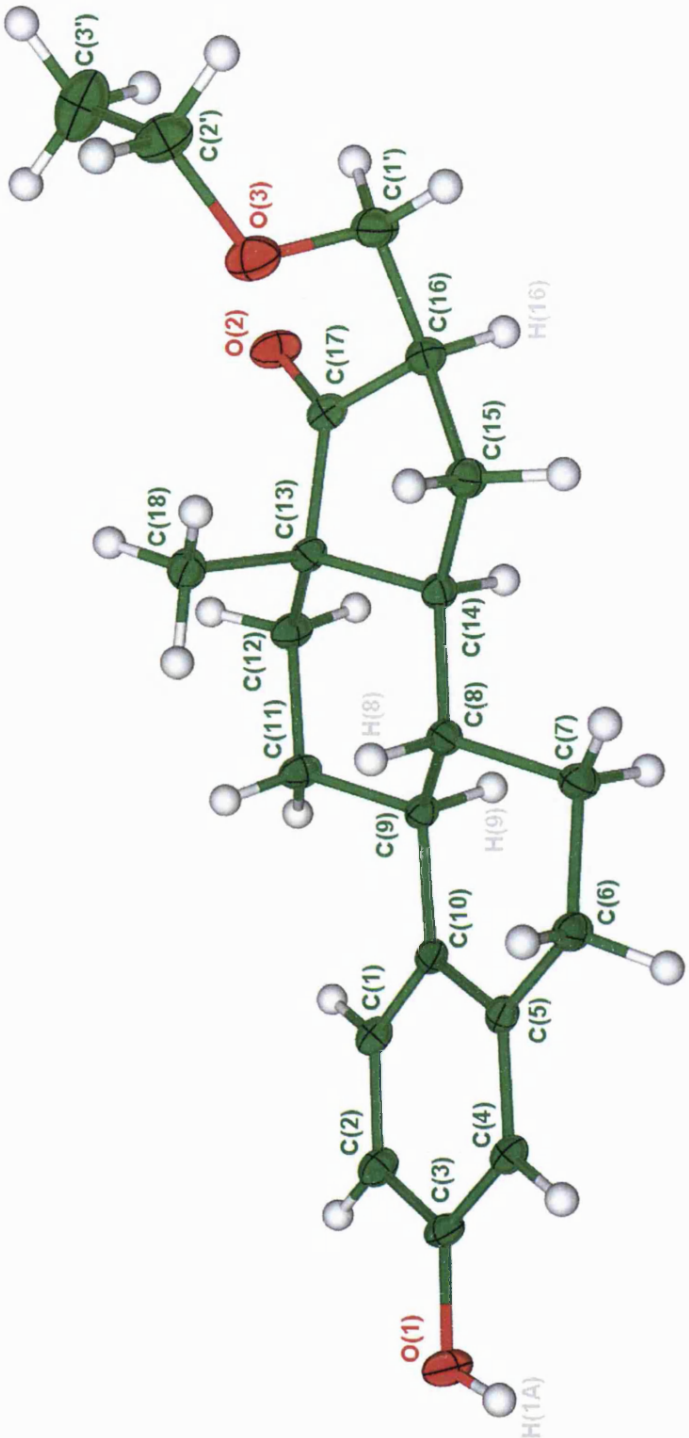
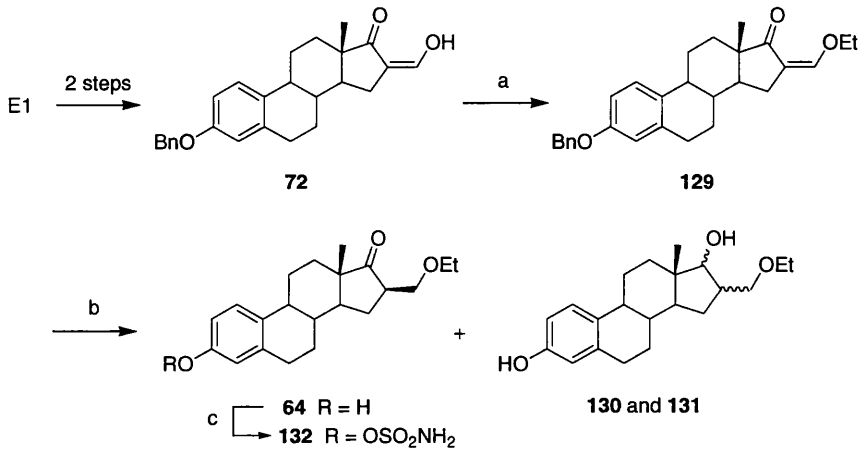


Figure 3.39 ORTEX[173] plot of the X-ray crystal structure of **64**. Ellipsoids are shown at the 30% probability level.

Building on the synthesis of several 16-hydroxymethylene derivatives of E1 (see section 3.2), it was anticipated that under catalytic hydrogenation conditions these enols would be selectively converted into the 16β-alcohols by *syn*-addition of two hydrogens on the less sterically hindered face of the double bond. Similar effects of angular methyl groups towards reduction of alkoxymethylene moieties have been observed in the literature.[264] Given that alkenes are more reactive than other functional groups toward catalytic hydrogenation, we did not expect any side-product to be formed as the result of the reduction of the C17 carbonyl.



Scheme 3.16 Alternative pathway for the synthesis of **64**. (a) K_2CO_3/DMF, EtI; (b) Pd/C, H_2, MeOH/THF; (c) $ClSO_2NH_2$/DMA.

To access **64** in a minimum number of steps, we decided to first alkylate the enol function of the benzylated precursor **72** to form the ethoxymethylene derivative **129**. Subsequent hydrogenation was expected to reduce the double bond at C16 as well as cleave the protecting group at C3 (Scheme 3.16). The intermediate **72** was obtained in 61% overall yield in two steps from E1. Treatment of this enol according to a literature procedure[152,257] with potassium carbonate and ethyl iodide in acetone easily afforded **129** in 80% yields. Unexpectedly, hydrogenation of the latter for 48 hours at room temperature gave a mixture of three products, all of which were debenzylated at C3. Separation of the crude mixture by flash chromatography afforded **64** in 46% yield as the least polar compound. Examination of the ^1H NMR spectra of the two more polar products isolated from the column indicated that they corresponded to 16-ethoxymethyl derivatives of E2 (**130** and **131** in increasing polarity), which was

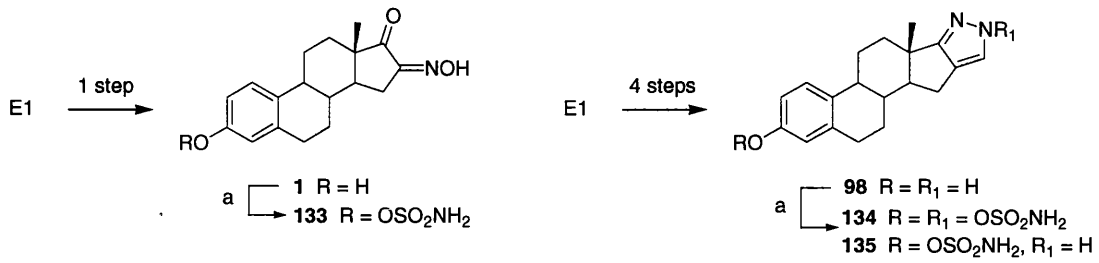
corroborated by mass spectrometry. Because of the new two chiral centres at C16 and C17, these two compounds could theoretically be any of four possible isomers. Determination of the coupling constants between C16-H and C17-H was expected to help assigning the structures of **130** and **131** since in the cyclopentane ring system, the coupling constant between *cis*-protons is higher than for *trans*-protons. In **130**, C17-H and C16-H were coupled with a constant of 9.9 Hz, however, we were unable to compare this value with the corresponding coupling constant in **131**, as the signal for C17-H was within the multiplet for the methylene protons of the ethoxymethyl side-chain, in the δ 3.3-3.6 region. Since the reduction was expected to occur preferably on the α-face, it can be surmised that isomer **131**, which was present in larger quantities (19% yield vs. 9% for **130**), contains the β,β arrangement of the C16 and C17 substituents. Compound **64** was then sulfamoylated following a literature procedure[171] using sulfamoyl chloride in DMA to give **132** in quantitative yield.

The determination of the stereochemistry of **130** and **131** was not further investigated given that these compounds were formed in relatively minor proportions. However, the formation of side-products during the hydrogenation step suggests that this pathway (Scheme 3.16) for the synthesis of **64** is not ideal, although it allowed synthesis of the product in larger quantities than in the previous method. Another sequence might be developed to access **64**, or alternatively, it might be useful to investigate the hydrogenation conditions, in order to prevent reduction at C17. Monitoring of the hydrogenation reaction by TLC however indicated that for shorter reaction times, some starting material was still present, while longer exposure to H_2 and the catalyst lead to reduction of the ketone at C17.

ii) Other derivatives

Access to the remaining target compounds **133** and **135** was achieved through sulfamoylation of the respective phenolic precursors **1** and **98** (Scheme 3.17). Reaction of **1** with sulfamoyl chloride in DMA gave **133** as a single product, however, loss of material through purification led to a yield of 37%. Comparison of the ^{1}H NMR spectrum of and **1** and **133** confirmed that sulfamoylation occurred at the phenolic end and not at the oxime moiety. In **133**, the oxime signal (δ 12.41) was

unchanged (cf. **1**), while the peak for C3-OH was not seen. The sulfamate NH_2 group resonated at δ 7.92.



Scheme 3.17 Synthesis of the sulfamoylated derivatives **133** and **135**. (a) $ClSO_2NH_2$/DMA.

Treatment of **98** under the same conditions gave a mixture of two products of distinct polarities, which were identified as **134** and **135**. The more polar product **135** was obtained with a yield of 57% while **134** was formed in smaller quantities and was recovered in 32% yield. Compound **134** was identified as a bis-sulfamoylated product on the basis of ^1H NMR and mass spectrometry evidence. The signals for the NH_2 group of each sulfamate moiety were seen at δ 7.88 and δ 8.30 in the ^1H NMR spectrum, and the peak for the heterocyclic proton (C5′-H) was observed at δ 7.70 (Figure 3.40).

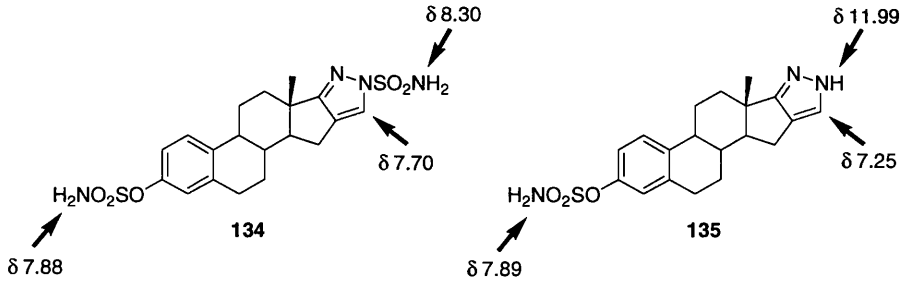


Figure 3.40 ^1H NMR shifts for characteristic protons in **134** and **135** (DMSO-d_6, 400 MHz).

When a sample of **134** was kept for several hours in DMSO-d_6 at room temperature and a new spectra collected we observed that:

(a) one sulfamate peak (δ 8.30) decreased in intensity while the other (δ 7.88) was unchanged;

(b) the signal for C5′-H disappeared;

(c) a peak at δ 7.25 appeared.

This was indicative of the rapid degradation of **134** into **135**, for which C5'-H and NH_2 resonated respectively at δ 7.25 and δ 7.89 (Figure 3.40). Cleavage of the sulfamate at the heterocyclic nitrogen (vs. C3) in aprotic polar solvents was strongly supported by the shift observed for C5'-H before and after degradation. In **134**, the sulfamate group was assumed to be directed at the least hindered nitrogen (N1') because of the steric effects of the 18-methyl group, although we do not have any experimental evidence to support this assumption.

3.4.3 Results and discussion

i) Inhibition of 17β-HSD type 1 and STS in vitro

Compound **133** was tested for its activity against both enzymes in separate assays. While 17β-HSD type 1 and type 2 activities were assessed in T-47D and MDA-MB-231 cells respectively, STS activity was assessed in placental microsomes. Table 3.10 summarises the data collected. The activity of **1**, the precursor of **133** and potent 17β-HSD type 1 inhibitor, is also reported here as both compounds were tested in the same assay. 667 COUMATE was used as a reference for STS inhibition.

Table 3.10 Inhibition of 17β-HSD type 1 (and type 2) and STS by **133** and reference compounds.

	17β-HSD		STS
Compound	17β-HSD type 1	17β-HSD type 2	IC_{50} (nM)
133	47.1 ± 0.9	7.6 ± 2.4	414
1	95.1 ± 1.0	5.0 ± 4.2	nd
667 COUMATE	nd	nd	5.5

The inhibitory data for **133** indicate that the structural modifications introduced at C3 were detrimental to its activity against 17β-HSD type 1. At a concentration of 10 μM, **133** inhibits only half of the activity of 17β-HSD type 1 while its phenolic precursor **1** achieved a 95% inhibition. With an IC_{50} of 414 nM in placental microsomes, **133** was also a 75 times less potent STS inhibitor than 667 COUMATE.

Although clearly not as effective as 667 COUMATE, **133** remains a moderately potent inhibitor of STS.

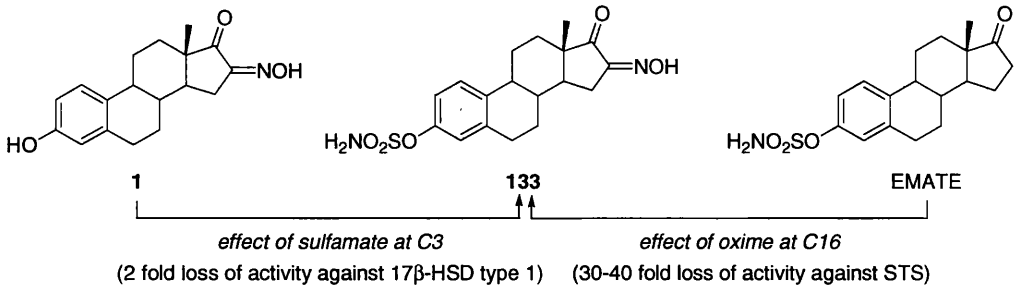


Figure 3.41 Structures of **1**, **133** and EMATE and comparison of the structural features influencing the biological activity.

When comparing the activities of **1** and **133** (Figure 3.41), it is clear that the introduction of a sulfamate moiety at C3 contributed to decreasing the activity against 17β-HSD type 1. This was to some extent expected, as we previously stressed on the importance of the interactions of the 3-hydroxyl moiety of the A-ring with key residues of 17β-HSD type 1 active site. This is exemplified by the fact that most steroidal inhibitors of 17β-HSD type 1 developed so far possess this feature.[121] A sulfamate group can potentially hydrogen bond to the polar residues surrounding the A-ring, yet, **133** only inhibits 47% of 17β-HSD type 1 activity at 10 μM. It is possible that despite favourable interactions of the sulfamate group in the active site, the D-ring moiety imparts negatively to the activity. The 16-oxime, which in **1** interacts favourably in the active site, may be shifted towards a more hydrophobic area to accommodate the increased bulk at the A-ring end. The loss of favourable interactions, and possibly consequent electrostatic repulsions, might explain the weak inhibitory activity of **133** against 17β-HSD type 1.

Concerning the inhibition of STS, it is believed that most of the favourable interactions can be attributed to the sulfamate moiety, yet **133** remains considerably less potent than 667 COUMATE. We have extensively discussed in Chapter 2 the importance of hydrophobic interactions of moieties situated on the D-ring of an E1/E2 or EMATE template. Interestingly, **133** is structurally derived from EMATE,

and the activity of both compounds against STS can be compared (Figure 3.40). EMATE, which is known to be 2-3 times less potent than 667 COUMATE *in vitro*, is therefore 30-40-fold more effective than **133** at inhibiting STS activity. The presence of the oxime moiety at C16 therefore decrease the activity of the derivative, which corroborates the aforementioned requirement for a hydrophobic group at this position or around the D-ring.

ii) Estrogenicity in vitro

The ability of some derivatives to provoke an estrogenic response was assessed *in vitro*, in a luciferase reporter gene assay. Figures 3.42 and 3.43 give a representation of the estrogenic activity for the sulfamates **132** and **133**, as well as for their respective phenolic precursors **64** and **1** at four different concentrations. The activity of **1**, which was discussed in section 3.3 is included for comparison, and E2 was used as a reference in all assays

All the compounds, except **132**, displayed some estrogenic activity at a concentration of 100 nM. While compound **1** was the most estrogenic derivative, its sulfamate analogue **133** had a weaker activity at the ER. It was a clear agonist of the ER at 100 nM, with an activity higher than 10 pM of E2. Both the 16β-ethoxymethyl derivatives **64** and **132** had no or weak estrogenic activity. In particular, **132** was not an agonist of the ER at 100 nM while **64** was a weak agonist at this concentration. The introduction of an ethoxymethyl at C16 therefore provoked a marked reduction of the intrinsic estrogenicity of E1, which was generally found to be estrogenic at 10 nM in our assays.

As predicted, a sulfamate moiety at C3 contributed to a weaker estrogenicity of the corresponding derivatives. Extensive studies on ER binding affinities of various steroids[37] and resolution of the crystal structure of the LBD of ER in complex with agonists and antagonists [41-43] have suggested that the 3-hydroxyl group was crucial for high affinity binding at the ER. We therefore expected the steroidal sulfamates **132** and **133** to be weaker agonists than their phenolic precursors **64** and **1**. This was particularly striking with the 16-oxime derivatives **1** and **133** which were separated

by a 1000-fold difference in estrogenic concentrations (100 pM and 100 nM respectively). This effect was also observed in **64/132**, with **132** being totally devoid of estrogenic activity.

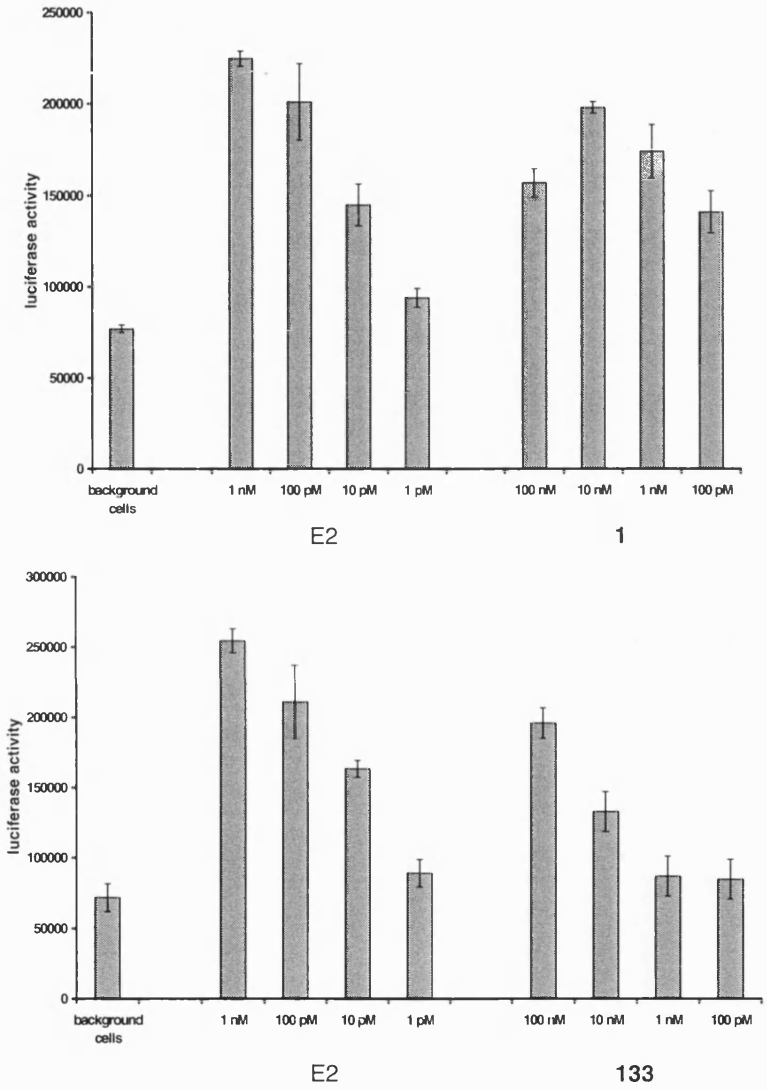


Figure 3.42 Estrogenicity *in vitro* for the sulfamoylated derivative **133** and its phenolic precursor **1**.

Clearly, an oxime moiety at C16 did not prevent high affinity binding to the ER, as exemplified by the high agonistic activity of **1**. This contrasts with previous reports where the ER was found to have a limited tolerance for polar moieties at C16α,[152,257] although the difference in orientation between a moiety at C16α and the polar group in **1** might be sufficient to explain its estrogenic activity. In contrast, the effects of an

ethoxymethyl side-chain at C16β were encouraging, with **64** and **132** being considerably less estrogenic than **1** and **133**. Interestingly, the highly potent 17β-HSD type 1 inhibitor **64** (IC_{50} = 320 nM in T-47D cells) was a poor agonist of the ER at 100 nM, which may indicate a low affinity binding of such compounds to the ER. This in turn suggests the potential of ether-linkage in the D-ring modifications.

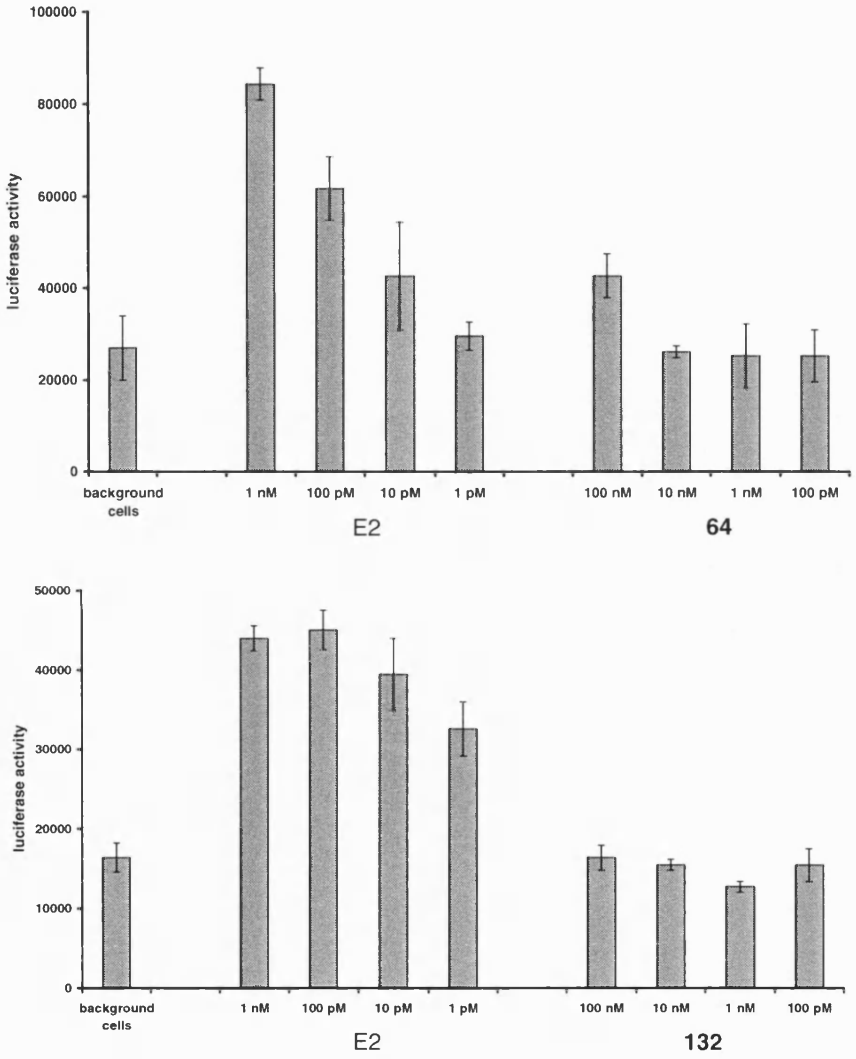


Figure 3.43 Estrogenicity *in vitro* for the sulfamoylated derivative **132** and its phenolic precursor **64**.

3.4.4 Conclusion

Agents that inhibit more than one enzyme of the steroidogenic pathway may contribute to a more efficient blockade of estrogen production in tumour cells. Recently, some compounds were shown to successfully inhibit both STS and aromatase activity *in vitro* and *in vivo*.[111] To verify if this concept is also applicable to the dual inhibition of 17β-HSD type 1 and STS, a small set of novel compounds was synthesised. By introducing the pharmacophore for STS inhibition in known 17β-HSD type 1 inhibitors, we hoped to engineer into these compounds some inhibitory activity against STS, while retaining their 17β-HSD type 1 inhibitor properties.

Compounds **132**, **133** and **135** were synthesised in one step from their phenolic precursors. *In vitro*, the 16-oximino derivative **133** was found to be a poor inhibitor of 17β-HSD type 1 activity with moderate potency against STS. Clearly, the modification at the A-ring end reduced the activity of the derivative against 17β-HSD type 1, while the moiety at the D-ring end did not contribute sufficiently to favourable interactions in STS active site. Examination of the estrogenicity of **132** and **133** compared with that of their precursors confirmed that the presence of a sulfamate moiety was correlated to reduced estrogenicity.

More biological data are however needed to conclude whether the approach chosen might lead to potent dual inhibitors of 17β-HSD type 1 and STS. In particular, A-ring sulfamates with a variety of D-ring features need to be designed and synthesised in order to assess whether a compromise can be reached between the structural requirements responsible for high affinity binding in both active sites.

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Summary

Estrogens have a well-established role in the aetiology of breast cancer. They directly support the growth and development of hormone-dependent forms of carcinoma and therapeutic strategies aimed to deprive the tumours of estrogens by either antagonising the ER, or by blocking their biosynthetic pathways, have seen an accelerated development in the past decade. In particular, steroidogenic enzyme inhibitors represent an attractive class of compounds that can potentially deplete circulating and tissue levels of active estrogens. While aromatase inhibitors are currently used in the treatment of HDBC, drugs that inhibit STS or 17β-HSD type 1 have not yet progressed to clinical trials. A major difficulty in the design of agents to treat hormone-dependent diseases lies in the requirement for a non estrogenic profile of the drug. A number of strategies have been adopted to design and synthesise novel inhibitors of STS and 17β-HSD type 1 that would be highly active while being devoid of estrogenic activity.

Attempts to develop potent inhibitors of STS were met with success. EMATE (Figure 3.44), the highly active, albeit estrogenic, landmark inhibitor was used as a structural basis for the design of a new template. To explore the potential of hydrophobic interactions around the steroidal D-ring, while possibly overcoming the intrinsic estrogenicity of EMATE, structural modifications were envisaged. The cyclopentanone steroidal D-ring was transformed into a piperidinedione moiety while the active pharmacophore for STS inhibition, an aryl-*O*-sulfamate moiety, was retained. The synthetic strategy developed afforded the resulting 3-sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide template in a minimum number of steps, and facile introduction of diversity rapidly yielded a series of *N*-alkylated analogues. Biological evaluation of these novel derivatives indicated that not only most of the compounds synthesised were potent, non estrogenic inhibitors of STS, but two analogues (*N*-propyl **35** and *N*-pyridin-3-ylmethyl **43**, Figure 3.44) were found to be 18 times more active than EMATE *in vitro*, with IC_{50}s of 1 nM in placental microsomes. The importance of hydrophobic interactions in the active site was confirmed (e.g. *N*-benzyl **44** and *N*-^{t}butylbenzyl **42** had IC_{50}s below 25 nM) however, the corresponding pocket did not tolerate long linear side-chains (IC_{50}s above 150 nM for C_4-C_6 side-chains).

In an effort to rationalise the activity of such compounds, molecular modelling studies using the recently disclosed three dimensional structure of human STS were carried out. Key interactions contributing to high affinity binding of the D-ring modified inhibitors in the active site were examined. The pseudo imido-carbonyl at C16 was proposed to hydrogen bond to an amino acid of the active site (Arg98), however interactions of the sulfamate moiety with catalytic residues and the metal ion Ca^{2+} seemed to dictate one major binding mode for the inhibitors upon docking. A QSAR was established for this series of D-ring modified analogues and its predictive power confirmed. It is anticipated that this QSAR will assist in the design of future potent inhibitors.

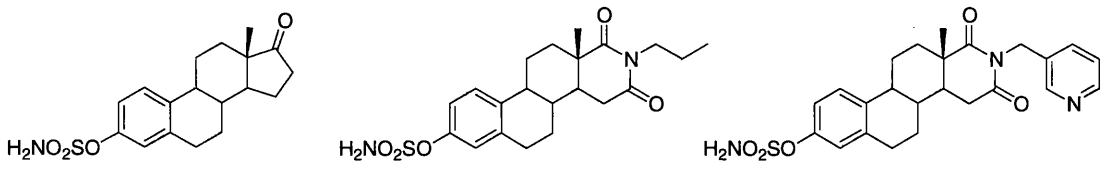
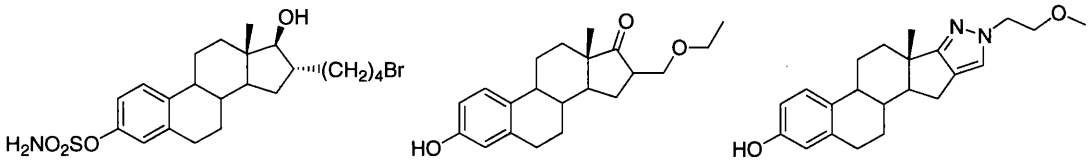


Figure 3.44 Structure and IC_{50} values for EMATE and D-ring modified STS inhibitors (IC_{50} values determined in placental microsomes).

In vivo evaluation of **35** and **43** confirmed their potential as future drug candidates. Both compounds were found to inhibit rat liver sulfatase activity by 99% when treated orally at a dose of 10 mg/kg, while being devoid of estrogenic activity. Further investigations need to be carried out to determine in particular whether such compounds inhibit the enzyme in an irreversible manner.

The design of 17β-HSD type 1 inhibitors faced the difficulty that relatively few potent compounds have been reported and consequently no pharmacophore was identified, despite the resolution of the crystal structure of this enzyme nearly 10 years ago. The existence of several isotypes has added a level of complexity to the work on inhibition since selectivity of drug action has to be achieved when targeting 17β-HSDs. The logical approach to the development of inhibitors was therefore to carry out substrate-based design.

In a limited study on mechanism-based inhibitors of 17β-HSD type 1, we aimed to identify new E2 derivatives that would behave as potential substrates for the enzyme and be converted *in situ* to affinity alkylators. *In vitro* testing of the resulting 16-alkylidene compounds led to inconclusive results and their full potential is expected to be assessed after optimisation of the biological assay. Unexpectedly, 16-ethoxymethyl-estrone (**64**, Figure 3.45) emerged from these studies as a highly potent inhibitor. Molecular modelling investigations on 16-methylene-estrone, a known enzyme-generated inhibitor provided interesting insight into the mechanism of inhibition. A cysteine residue (Cys185) of the active site was proposed as the nucleophile potentially involved in irreversible inactivation of the enzyme.



1.18 IC_{50} = 460 nM

64 IC_{50} = 320 nM

88 IC_{50} = 530nM

Figure 3.45 Structure and IC_{50} values for 17β-HSD type 1 inhibitors (IC_{50} values determined in T-47D cells except for **1.18**, in human placental cytosol).

Despite the lack of a pharmacophore for 17β-HSD type 1 inhibition, the D-ring of E1/E2 proved to be a crucial location for the introduction of functionalities able to block enzymatic activity. Most of the potent 17β-HSD type 1 inhibitors reported are C16 or C17 modified E1/E2 derivatives, 16α-bromobutyl-estradiol (**1.18**, Figure 3.45) being among the most active. Further to the identification of the synthetic intermediates 16-oximino- and 16-hydroxymethylene-estrone (**1** and **97**) as potent inhibitors of 17β-HSD type 1 (respective IC_{50}s of 1.10 μM and 0.11 μM in T-47D cells), a series of D-ring fused heterocyclic derivatives of E1 were synthesised as putative bioisosteres. To probe the active site in several directions, a 16,17-fused pyrazole derivative of E1 was functionalised at the 1′, 2′ and 5′ position and the synthetic routes developed efficiently afforded the final compounds via common intermediates. *N*-Alkylation of the pyrazole nucleus yielded isomers which were separated and their regiochemistry unambiguously assigned. *In vitro* evaluation afforded an SAR in which the 1′-alkylated (vs. 2′-alkylated) compounds were more

potent. Substituents able to make polar interactions were preferred and the *N*-methoxyethyl analogue **88** (Figure 3.45) emerged as a promising compound, being also devoid of estrogenic activity *in vitro*.

Investigations aimed to identify other positions of the steroidal nucleus (E1/E2) available for functionalisation were not entirely successful. SAR studies in a series of keto/oximino derivatives of E1 at the 6, 16, 17 or a combination of these positions provided interesting results, however no synergy was observed when substituents were combined suggesting that they might be involved in mutually exclusive interactions at the active site. *In vitro* activity of 6-oxo-estrone **120** (IC_{50} = 340 nM) confirmed the potential of the 6-position for the introduction of a small polar moiety, as suggested by the identification of Ser222 in the vicinity of the B-ring in an enzyme-E2 complex. Structural modifications need to be implemented to overcome the estrogenicity of some of these derivatives.

Building on our investigations in the field of steroidogenic enzymes inhibition, a preliminary study focused upon the design of dual inhibitors of STS and 17β-HSD type 1 was initiated. Introduction of the pharmacophore for STS inhibition in potent 17β-HSD type 1 inhibitors proved easy and the validity of this approach needs to be further assessed.

A useful template for the design of potent STS inhibitors was proposed and two highly active non estrogenic derivatives with *in vivo* activity emerged from these studies. Several new lead structures have been identified for 17β-HSD type 1 inhibition and it is expected that their optimisation might result in potential drug candidates. These investigations should contribute to further the development of steroidal inhibitors of STS and 17β-HSD type 1 for the treatment of HDBC.

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Experimental

5.1 General Methods

All chemicals were either purchased from Aldrich Chemical Co. (Gillingham, UK) or Lancaster Synthesis (Morecambe, U.K.). All organic solvents of A. R. grade were supplied by Fisher Scientific (Loughborough, U.K.). Anhydrous *N,N*-dimethylformamide (DMF) and *N,N*-dimethylacetamide (DMA), used for *N*-alkylations and sulfamoylation reactions, were purchased from Aldrich and were stored under an atmosphere N_2 after use. Sulfamoyl chloride was prepared by an adaptation of the method of Appel and Berger[265] and was stored at 5°C under an atmosphere N_2 as a solution in toluene as described by Woo *et al.*[266] An appropriate volume of this solution was freshly concentrated under reduced pressure immediately before use.

E1 was purchased from Sequoia Research Products (Oxford, UK). E1S and NADPH (tetrasodium salt) were supplied by Sigma Chemical Co. (Poole, UK). Radiolabelled E1 and E2 (^{3}H and ^{14}C) as well as ^{3}H-E1S were purchased from New England Nuclear (Boston, USA) or Amersham Biosciences UK Limited (Amersham, U.K.).

Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium sheets silica gel 60 F_{254}). Product(s) and starting material(s) were detected by either viewing under UV light and/or treating with an ethanolic solution of phosphomolybdic acid followed by heating. Flash column chromatography was performed on silica gel (Sorbsil/Matrex C60); for compounds **78** and **79**, Flashmaster™ was used with prepacked columns of 10 g. IR spectra were recorded on a Perkin-Elmer Spectrum RXI FT-IR as KBr discs and peak positions are expressed in cm^{-1}. ^{1}H NMR and *DEPT*-edited ^{13}C NMR spectra were recorded with a JMN-GX 400 NMR spectrometer at 400 and 100 MHz respectively, and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS) as an internal standard. The following abbreviations are used to describe resonances in ^{1}H NMR and ^{13}C NMR spectra: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet etc. Chemical shifts for AB systems were approximated by taking the middle of each doublet and the corresponding coupling constant labelled J_{AB} or J_{BA}. HPLC analyses were performed on a Waters

Millenium 32 instrument equipped with a Waters 996 PDA detector. A Waters Radialpack C18, 8×100 mm column was eluted with a MeOH/H_2O gradient at 2 mL/min. Preparative HPLC was performed on a Waters Nova-Pak C18, 25×100 mm column eluted with a MeOH/H_2O gradient at 20 mL/min. Mass spectra were recorded at the Mass Spectrometry Service Center, University of Bath. FAB-MS were carried out using *m*-nitrobenzyl alcohol (NBA) as the matrix. Elemental analyses were performed by the Microanalysis Service, University of Bath. Melting points were determined using a Reichert-Jung Thermo Galen Kofler block and are uncorrected. The X-ray crystallographic studies of **35**, **62**, **64** and **84** were carried out by Dr. M. F. Mahon in the Department of Chemistry, University of Bath, on a kappa CCD diffractometer with area detector.

Molecular modelling was performed in collaboration with Mr. J.J. Robinson on a Silicon Graphics Octane 2. The molecular operating environment (MOE) from the Chemical Computing Group Inc. was used for model building and QSAR determination. The three dimensional molecular viewer ViewerLite 5.0 from Accelrys Inc. or MOE were used to construct diagrams. Docking studies and scoring were performed using Gold® v2.1.

5.2 Biological assays

In vitro estrogenicity assays were performed at the Cancer Research UK Oncology Unit, Southampton General Hospital, Southampton by Drs. L. Wood and G. Packham. Metabolic studies were carried out at the Magdalen Centre, Oxford Science Park, Oxford in collaboration with Dr. D.J. McCormick. All other assays were performed at the Department of Endocrinology and Metabolic Medicine, Imperial College School of Medicine, St. Mary's Hospital, London by and in collaboration with Dr. A. Purohit and Prof. M. J. Reed.

5.2.1 *In vitro* assays

Inhibition of STS activity

STS inhibition was assessed as described previously.[88] Placental microsomes (100000 g fraction) were incubated with ^{3}H-E1S adjusted to a final concentration of 20 μM with unlabeled substrate, in the absence or presence of the inhibitor (0.1 nM-1.0 μM). Product formed was isolated from the mixture by extraction with toluene (4 mL), using ^{14}C-E1 to monitor procedural losses. The mass of ^{3}H-E1 produced was calculated from the ^{3}H counts detected and recovery of ^{14}C-E1.

Inhibition of 17β-HSD type 1 activity

T47-D human breast cancer cells were incubated with ^{3}H-E1 at a concentration of 5 pmol per flask, in the absence or presence of the inhibitor (0.1 nM-1μM). After incubation of the substrate ± inhibitor for 30 minutes at 37°C, the products were isolated from the mixture by extraction with Et_2O (4 mL), using ^{14}C-E2 to monitor procedural losses. Separation of ^{3}H-E2 from the mixture was achieved using preparative TLC (DCM/EtOAc, 4:1) and the mass of ^{3}H-E2 produced was calculated from the ^{3}H counts detected and recovery of ^{14}C-E2.

Reversibility assay

T-47D cells were pretreated with 10 μM or 20 μM of the inhibitor at 37°C overnight. The flasks were either washed (5 times) with assay medium or had their assay medium removed. Each group of flasks (washed and not washed) were then treated with either ^{3}H-E1 or ^{3}H-E1 and the inhibitor (10 μM or 20 μM), incubated for 30 minutes at 37°C and processed as normal.

Inhibition of 17β-HSD type 2 activity

MDA-MB-231 human breast cancer cells were incubated with ^{3}H-E2 at a concentration of 5 pmol per flask, in the absence or presence of the inhibitor (0.1 nM-1μM). After incubation of the substrate ± inhibitor for 30 minutes at 37°C, the products were isolated from the mixture by extraction with Et_2O (4 mL), using ^{14}C-E1 to monitor procedural losses. Separation of ^{3}H-E1 from the mixture was achieved

using preparative TLC (DCM/EtOAc, 4:1) and the mass of ^{3}H-E1 produced was calculated from the ^{3}H counts detected and recovery of ^{14}C-E1.

Inhibition of cell growth

This was assessed as described previously.[94] MCF-7 human breast cancer cells (5000 per well) were incubated in the absence or presence of the test compound (1 nM to 100 μM) for 4 days. The effect on cell growth was determined using a Cell Titer 96 proliferation assay. This assay uses a tetrazolium dye to measure the number of metabolically active cells.

Estrogenicity and anti-estrogenicity assay

A luciferase reporter gene assay was used to measure the effect of ER binding compounds on gene expression through the activation of ERE. MCF-7 cells (ER positive) were transfected with a pERET81luc reporter construct. After 24 hours, the cells were exposed to either the candidate estrogen compound (for estrogenicity measurement) or the candidate estrogen compound and E2 (for anti-estrogenicity measurement). After a further 24 hours, the cells were assayed for luciferase activity using a bioluminescence assay. To control for transfection efficiency, cells were cotransfected with a control reporter construct containing the β-gal gene, and β-galactosidase activity was measured using a colorimetric assay.

Metabolic studies

Hepatic microsomes (47 μL of a 20 mg/mL sample) were incubated with the test compound (final concentration 18 μM) for 10 minutes at 37°C. An aliquot, corresponding to time 0 was taken and added to an internal standard before addition of NADPH (0.1 mL of a 3.6 mg/mL standard solution). Samples were then taken at times 10, 30 and 60 minutes and added to the internal standard. Analysis of the samples was performed by HPLC. Peak area ratio was determined for the test compound and the internal standard, and the ratio (test compound/internal standard) was calculated for each time point. The data were used to calculate the half life ($t_{1/2}$) and clearance (Cl) for each compound.

5.2.2 *In vivo* studies

Ovariectomised female Wistar rats (200 g) were obtained from Charles River (Kent, UK) and kept under conditions meeting institutional requirement with free access to food and water. Groups of rats, with 3 rats in each group, were treated with EMATE (50 μg /kg, s.c.) or **35** or **43** (10 mg/kg, p.o.) once daily for a 5-day period. Control groups of rats were given vehicle (propylene glycol, 200 μL, p.o.).

Uterotrophic estrogenicity study

Animals were killed using an approved procedure 24 hours after administration of the last dose of compound, and uteri were excised of fat and weighed. Total body weights of the rats were also recorded, and the results were expressed as uterine weight×100/total body weight.

Inhibition of STS activity

Samples of liver tissue obtained from animals 24 hours after administration of the last dose of the drug, were immediately frozen on solid carbon-dioxide and stored at -20°C until assay.[89] After homogenisation and centrifugation, aliquots of the resulting tissue supernatants were incubated with ^{3}H-E1S adjusted to a final concentration of 20 μM with unlabelled E1S, for 1 hour at 37°C. Product formed was isolated from the mixture by extraction with toluene (4 mL), using ^{14}C-E1 to monitor procedural losses. The mass of ^{3}H-E1 produced was calculated from the ^{3}H counts detected and recovery of ^{14}C-E1. The protein concentration in supernatants was measured by the method of Bradford.[267]

Statistics

The significance of differences in uterine weight and steroid sulfatase activity in tissues from control and treated animals was assessed using Student's *t* test.

5.3 General synthetic procedures

5.3.1 Preparation of sulfamoyl chloride

Freshly distilled formic acid (6 mL, 150 mmol) was added dropwise to a stirred solution of chlorosulfonyl isocyanate (25 g, 150 mmol) in freshly distilled toluene (150 mL) at 0°C under an atmosphere of N_2. The resulting white suspension was stirred overnight at room temperature and concentrated under reduced pressure to give a light brown crude of sulfamoyl chloride. The product was dissolved in freshly distilled toluene (150 mL) and filtered under an atmosphere of N_2 using a cannula into a 250 mL volumetric flask. The volume of toluene was then completed to 250 mL, giving a standard solution (ca. 0.70 M) of sulfamoyl chloride which was stored in the fridge under N_2.

5.3.2 Method 1. Synthesis of alkylated derivatives 6-16 and 30

NaH (60% dispersion in mineral oil, 1.2 eq.) was added to a stirred solution of **5** in anhydrous DMF (15 mL) at 0°C under an atmosphere of N_2. After 20 minutes of stirring, the parent alkylating agent (2 eq.) was added and the resulting mixture was stirred at room temperature. The reaction was monitored by TLC and quenched with H_2O (50 mL) at completion. The organics were extracted with EtOAc (2×50 mL), washed with brine (4×25 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography.

5.3.3 Method 2. Hydrogenolysis

A suspension of Pd-C 10% (ca. 50% w/w of substrate used) in THF (2 mL) was added to a solution of the starting material in MeOH/THF. The resulting suspension was hydrogenated at room temperature using a hydrogen-filled balloon until TLC indicated completion. After removal of the supported catalyst by filtration through celite and concentration of the filtrate under reduced pressure, the product obtained was purified by flash chromatography and/or recrystallised.

5.3.4 Method 3. Sulfamoylation according to Okada *et al.*[171]

Anhydrous DMA was added to a freshly concentrated solution of sulfamoyl chloride (2.2 eq.) cooled to 0°C under an atmosphere of N_2. The phenolic substrate was then added and the resulting mixture was stirred at room temperature until TLC indicated completion. The mixture was then poured into cold brine (15 mL), and the organics were extracted with EtOAc (2×20 mL), washed with brine (5×20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography and/or recrystallised.

5.3.5 Method 4. Preparation of the sulfamates 39, 43, 51 and 60

NaH (60% dispersion in mineral oil, 1.2 eq.) was added to a stirred solution of the phenolic substrate in anhydrous DMF at 0°C under an atmosphere of N_2. After 15 minutes, sulfamoyl chloride (6 eq.) was added and the resulting mixture was stirred at room temperature until TLC indicated completion. The mixture was then poured into brine (20 mL), and the organics were extracted with EtOAc (2×20 mL), washed with H_2O (2×20 mL), brine (2×20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography and/or recrystallised.

5.3.6. Method 5. Synthesis of alkylated derivatives 74-81

NaH (60% dispersion in mineral oil, 1.5 eq.) was added to a stirred solution of **73** in anhydrous DMF at 0°C under an atmosphere of N_2. After 20 minutes of stirring, the parent alkylating agent (2 eq.) was added and the resulting mixture was stirred at room temperature. The reaction was monitored by TLC and quenched with H_2O (50 mL) at completion. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography.

5.4 Synthesis

5.4.1 D-ring modified derivatives of EMATE and precursors

16-Oximino-estrone (1)

E1 (200 mg, 740 μmol) was added to a stirred solution of ^{t}BuOK, freshly prepared by dissolving potassium metal (80 mg, 2.05 mmol) in 2 mL *tert*-butanol, under an atmosphere of N_2. The reaction mixture was stirred for 1 hour at room temperature before dropwise addition of isoamyl nitrite (180 μL, 1.34 mmol). The resulting deep red mixture was stirred overnight, then poured into H_2O (20 mL). The organics were extracted with Et_2O (2×20 mL) and the aqueous layer was acidified with AcOH glacial (10 mL) to give a light yellow precipitate. After standing for 2 hours, the solid was isolated by filtration to give **1** as a yellow powder (140 mg, 63%). For analysis, a sample was recrystallised from acetone to give white crystals: mp 223-225°C [lit.[160] (MeOH) 218-220°C]; IR (KBr) 3385 (NOH), 2920-2860 (aliph CH), 1735 (C=O), 1605-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.89 (3H, s, C-18-H_3), 1.30-2.85 (11H, m), 2.70-2.81 (2H, m, C-6-H_2), 6.46 (1H, d, J = 2.3 Hz, C-4-H), 6.52 (1H, dd, J = 8.3 Hz, J = 2.3 Hz, C-2-H), 7.05 (1H, d, J = 8.3 Hz, C-1-H), 9.05 (1H, s, exchanged with D_2O, OH) and 12.39 (1H, s, exchanged with D_2O, NOH); δ_C (DMSO-d_6, 100.4 MHz) 14.1 (q, C-18), 25.1 (t), 25.5 (t), 26.2 (t), 29.0 (t), 30.9 (t), 37.2 (d), 43.2 (d), 44.6 (d), 48.5 (s, C-13), 112.7 (d), 114.8 (d), 125.8 (d), 129.6 (s), 136.9 (s), 154.8 (s, C-3 or C-16), 155.2 (s, C-3 or C-16) and 204.6 (s, C=O); MS *m/z* (FAB+) 453.2 [30, $(M+H+NBA)^+$], 300.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 451.3 [38, $(M-H+NBA)^-$], 298.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 300.1596, $C_{18}H_{22}NO_3$ requires 300.1600. Found: C, 71.30; H, 7.08; N, 4.35. $C_{18}H_{21}NO_3.(AcOH)_{1/6}$ requires: C, 71.17; H, 7.06; N, 4.53%.

3-Acetoxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (2)

A suspension of **1** (150 mg, 501 μmol) in a mixture of 4.5 mL of AcOH glacial and 7.5 mL of acetic anhydride was heated to reflux under an atmosphere of N_2 for 20 hours. The solvent mixture was then removed under reduced pressure and H_2O (20 mL) added. After cautious addition of 10% aq. NaOH (2-3 drops), the organics were

extracted with EtOAc (2×20 mL), washed with H_2O (2×15 mL), brine (2×15 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography ($CHCl_3$) to give **2** as a light yellow solid (97 mg, 57%): mp 189-193°C [lit.[160] (acetone) 196-198°C]; IR (KBr) 3205 (NH), 2940-2860 (aliph CH), 1760, 1725, 1690 (C=O), 1610-1495 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.11 (3H, s, C-18-H_3), 1.20-2.72 (11H, m), 2.77-2.84 (2H, m, C-6-H_2), 2.23 (3H, s, $COCH_3$), 6.81 (1H, d, J = 2.6 Hz, C-4-H), 6.87 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H), 7.32 (1H, d, J = 8.3 Hz, C-1-H) and 10.64 (1H, s, exchanged with D_2O, NH); δ_C (DMSO-d_6, 100.4 MHz) 16.0 (q, C-18), 20.7 (q, $COCH_3$), 24.8 (t), 24.9 (t), 28.8 (t), 32.2 (t), 32.5 (t), 37.3 (d), ~39 (s, under solvent peaks), 40.3 (d), 41.9 (d), 118.6 (d), 120.9 (d), 125.9 (d), 136.5 (s), 137.1 (s), 147.9 (s, C-3), 168.8 (s, $COCH_3$), 171.9 (s, C=O) and 178.7 (s, C=O); MS *m/z* (FAB+) 342.1 [100, $(M+H)^+$], 299.1 [40, $(M+H-Ac)^+$]; MS *m/z* (FAB-) 493.2 [34, $(M-H+NBA)^-$], 340.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 342.1705, $C_{20}H_{24}NO_4$ requires 342.1705.

3-Benzyloxy-estra-1,3,5(10)-triene-17-one (3)

NaH (60% dispersion in mineral oil, 0.68 g, 20.34 mmol) was added to a stirred solution of E1 (5.0 g, 18.49 mmol) in anhydrous DMF (50 mL), at 0°C under an atmosphere of N_2 and the resulting suspension was stirred for 1 hour. Benzyl bromide (2.42 mL, 20.34 mmol) was then added and the mixture was heated at 80°C for 4 hours. The reaction mixture was poured into ice/H_2O and the organics extracted with EtOAc (150 mL), washed with H_2O (4×50 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The pale yellow crude product was recrystallised from IPA to give **3** as white flaky crystals (4.73 g, 71%): mp 129-131°C [lit.[167] 126-127°C]; IR (KBr) 3100 (arom CH), 2950-2840 (aliph CH), 1730 (C=O), 1600, 1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.91 (3H, s, C-18-H_3), 1.41-2.54 (13H, m), 2.86-2.93 (2H, m, C-6-H_2), 5.04 (2H, s, OCH_2Ar), 6.73 (1H, d, J = 2.5 Hz, C-4-H), 6.80 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H), 7.20 (1H, d, J = 8.6 Hz, C-1-H) and 7.30-7.44 (5H, m, C_6H_5).

3-Benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-dioic acid (4)

A solution of iodine (7.6 g, 29.9 mmol) in MeOH (95 mL) and a solution of KOH (13.7 g, 245 mmol) in H_2O (25 mL) and MeOH (60 mL) were added dropwise and

alternately to a stirred solution of **3** (3.8 g, 10.5 mmol) in MeOH (1 L), at room temperature, so that the colour of the mix remains orange/brown. The addition was carried out over 45 minutes and the resulting light yellow solution was stirred overnight, after which it was concentrated under reduced pressure and poured into H_2O (800 mL). After acidification with 5M HCl, the organics were extracted with Et_2O (600 mL), washed with aq. $Na_2S_2O_3$ (4×100 mL), H_2O (4×100 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting yellow foam (4.54 g) was then dissolved in a solution of KOH (7.6 g, 136 mmol) in MeOH/H_2O (1:2, 228 mL) and heated to reflux for 4 hours. The resulting orange solution was poured into H_2O (800 mL), acidified with 5M HCl, and the organics extracted with EtOAc (300 mL). After washing with brine (4×200 mL), the organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow residue (4.32 g). The crude product was recrystallised from $CHCl_3$/Hexane (5:3) to give **4** as an off-white powder (3.25 g, 75%): mp 212-215°C [lit.[166] (aq. MeOH) 226-227°C]; IR (KBr) 3050-2650 (br, CO_2H, arom CH and aliph CH), 1700 (C=O), 1600-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.20-2.78 (11H, m), 2.72-2.76 (2H, m, C-6-H_2), 5.05 (2H, s, OCH_2Ar), 6.68 (1H, d, J = 2.5 Hz, C-4-H), 6.75 (1H, dd, J = 8.7 Hz, J = 2.5 Hz, C-2-H), 7.18 (1H, d, J = 8.7 Hz, C-1-H), 7.30-7.42 (5H, m, C_6H_5) and 12.14 (2H, s, exchanged with D_2O, CO_2H); δ_C (DMSO-d_6, 100.4 MHz) 15.4 (q, C-18), 25.8 (t), 26.5 (t), 29.7 (t), 35.8 (t), 36.1 (t), 40.7 (d), 41.8 (d), 42.5 (d), 46.2 (s, C-13), 68.9 (t, OCH_2Ar), 112.3 (d), 114.0 (d), 126.3 (d), 127.3 (2×d), 127.5 (d), 128.2 (2×d), 131.6 (s), 137.2 (2×s), 156.0 (s, C-3), 173.9 (s, CO_2H) and 178.6 (s, CO_2H); MS *m/z* (FAB+) 408.2 [41, M^+], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 408.1940, $C_{25}H_{28}O_5$ requires 408.1937.

3-Benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (5)

Urea (3.25 g, 54.11 mmol) and **4** (3.25 g, 7.96 mmol) and were heated at 180°C under an atmosphere of N_2 for 45 minutes. The resulting brown residue was crushed and acetone was added (200 mL) to give a brown suspension. This mixture was concentrated to ca. 100 mL, silica gel was added and the solvent was removed under reduced pressure to give an homogeneous light brown powder which was transferred onto a wet packed ($CHCl_3$) flash chromatography column. Elution with

$CHCl_3$/acetone (96:4) gave **5** as a white residue (2.75 g, 89%). An analytical sample was recrystallised from EtOH to give colourless needles: mp 225-226°C; IR (KBr) 3260 (NH), 2900-2870 (aliph CH), 1720 (C=O), 1700 (C=O), 1600-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.09 (3H, s, C-18-H_3), 1.20-2.72 (11H, m), 2.76-2.80 (2H, m, C-6-H_2), 5.05 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.5 Hz, C-4-H), 6.76 (1H, dd, J = 8.7 Hz, J = 2.5 Hz, C-2-H), 7.19 (1H, d, J = 8.7 Hz, C-1-H), 7.31-7.44 (5H, m, C_6H_5) and 10.63 (1H, s, exchanged with D_2O, NH); δ_C (DMSO-d_6, 100.4 MHz) 16.2 (q, C-18), 25.1 (t), 25.2 (t), 29.2 (t), 32.4 (t), 32.7 (t), 37.8 (d), 40.3 (d), 40.5 (s), 41.9 (d), 68.9 (t, OCH_2Ar), 112.2 (d), 114.1 (d), 126.0 (d), 127.3 (2×d), 127.4 (d), 128.2 (2×d), 131.5 (s), 137.0 (s), 137.1 (s), 156.0 (s, C-3), 172.1 (s, C=O) and 178.9 (s, C=O); MS *m/z* (FAB+) 390.2 [58, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 390.2059, $C_{25}H_{28}NO_3$ requires 390.2069. Found: C, 76.90; H, 6.99; N, 3.73. $C_{25}H_{27}NO_3$ requires: C, 77.09; H, 6.99; N, 3.60%.

3-Benzyloxy-*N*-methyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (6)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with methyl iodide (160 µL, 2.57 mmol) was complete within 45 minutes. The crude product was purified by flash chromatography ($CHCl_3$) to give **6** as a white residue (432 mg, 83%). An analytical sample was recrystallised from EtOH to give colourless crystals: mp 118-121°C; IR (KBr) 3160-3060 (arom CH), 2920-2870 (aliph CH), 1720 (C=O), 1670 (C=O), 1600-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.17 (3H, s, C-18-H_3), 1.26-3.00 (11H, m), 2.86-2.91 (2H, m, C-6-H_2), 3.15 (3H, s, N-CH_3), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.80 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.21 (1H, d, J = 8.6 Hz, C-1-H) and 7.32-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 16.6 (q, C-18), 25.6 (t), 25.8 (t), 27.0 (q, C-1′), 29.7 (t), 33.5 (t), 33.8 (t), 38.5 (d), 40.4 (d), 41.5 (s, C-13), 42.6 (d), 69.9 (t, OCH_2Ar), 112.6 (d), 114.5 (d), 126.1 (d), 126.3 (2×d), 127.7 (d), 128.4 (2×d), 131.5 (s), 137.0 (s), 137.2 (s), 156.8 (s, C-3), 171.8 (s, C=O) and 178.7 (s, C=O); MS *m/z* (FAB+) 404.4 [79, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 404.2217, $C_{26}H_{30}NO_3$ requires 404.2226. Found: C, 77.30; H, 7.22; N, 3.48. $C_{26}H_{29}NO_3$ requires: C, 77.39; H, 7.24; N, 3.47%.

3-Benzyloxy-*N*-ethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (7)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with ethyl iodide (205 μL, 2.57 mmol) was complete within 1 hour. The crude product was purified by flash chromatography ($CHCl_3$) to give **7** as a white residue (502 mg, 94%): mp 93-95°C; IR (KBr) 2975-2865 (aliph CH), 1715 (C=O), 1665 (C=O), 1605-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.11 (3H, t, *J* = 7.2 Hz, C-2′-H_3), 1.16 (3H, s, C-18-H_3), 1.31-2.98 (11H, m), 2.85-2.90 (2H, m, C-6-H_2), 3.81 (2H, m, N-CH_2), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, *J* = 2.7 Hz, C-4-H), 6.81 (1H, dd, *J* = 8.6 Hz, *J* = 2.7 Hz, C-2-H), 7.22 (1H, d, *J* = 8.6 Hz, C-1-H) and 7.30-7.44 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 13.1 (q, C-2′), 16.4 (q, C-18), 25.5 (t), 25.7 (t), 29.6 (t), 33.5 (t), 33.6 (t), 35.0 (t, C-1′), 38.5 (d), 40.2 (d), 41.3 (s, C-13), 42.4 (d), 69.8 (t, OCH_2Ar), 112.4 (d), 114.4 (d), 126.0 (d), 127.1 (2×d), 127.6 (d), 128.3 (2×d), 131.4 (s), 136.9 (s), 137.0 (s), 156.7 (s, C-3), 171.1 (s, C=O) and 178.0 (s, C=O); MS *m/z* (FAB+) 418.3 [90, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 417.2306, $C_{27}H_{31}NO_3$ requires 417.2304.

3-Benzyloxy-*N*-propyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (8)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with propyl iodide (250 μL, 2.57 mmol) was complete within 2 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **8** as a white residue (524 mg, 94%). An analytical sample was recrystallised from EtOH to give white crystals: mp 95-98°C; IR (KBr) 3035 (arom CH), 2960-2870 (aliph CH), 1720 (C=O), 1660 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.89 (3H, t, *J* = 7.6 Hz, C-3′-H_3), 1.16 (3H, s, C-18-H_3), 1.32-2.98 (13H, m), 2.83-2.88 (2H, m, C-6-H_2), 3.64-3.80 (2H, m, N-CH_2), 5.03 (2H, s, OCH_2Ar), 6.72 (1H, d, *J* = 2.7 Hz, C-4-H), 6.80 (1H, dd, *J* = 8.6 Hz, *J* = 2.7 Hz, C-2-H), 7.21 (1H, d, *J* = 8.6 Hz, C-1-H) and 7.30-7.44 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 11.4 (q, C-3′), 16.6 (q, C-18), 21.3 (t), 25.6 (t), 25.8 (t), 29.8 (t), 33.7 (t), 33.8 (t), 38.7 (d), 40.4 (d), 41.5 (s, C-13), 41.6 (t, C-1′), 42.5 (d), 70.0 (t, OCH_2Ar), 112.6 (d), 114.5 (d), 126.1 (d), 127.3 (2xd), 127.8 (d), 128.4 (2xd), 131.5 (s), 137.0 (s), 137.2 (s), 156.8 (s, C-3), 171.5 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 432.4 [88, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 432.2522, $C_{28}H_{34}NO_3$

requires 432.2539. Found: C, 77.60; H, 7.68; N, 3.26. $C_{28}H_{33}NO_3$ requires: C, 77.93; H, 7.71; N, 3.25%.

3-Benzyloxy-*N*-butyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (9)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with bromobutane (276 μL, 2.57 mmol) was complete within 4 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **9** as a white residue (513 mg, 90%). An analytical sample was recrystallised from EtOH to give white needles: mp 100-103°C; IR (KBr) 2960-2870 (aliph CH), 1720 (C=O), 1665 (C=O), 1615-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, t, J = 7.2 Hz, C-4′-H_3), 1.16 (3H, s, C-18-H_3), 1.28-2.99 (15H, m), 2.84-2.89 (2H, m, C-6-H_2), 3.75 (2H, m, N-CH_2), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.5 Hz, C-4-H), 6.81 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H), 7.22 (1H, d, J = 8.6 Hz, C-1-H) and 7.29-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 13.8 (q, C-4′), 16.5 (q, C-18), 20.1 (t), 25.5 (t), 25.7 (t), 29.6 (t), 30.0 (t), 33.5 (t), 33.7 (t), 38.5 (d), 39.7 (t, C-1′), 40.2 (d), 41.3 (s, C-13), 42.4 (d), 69.8 (t, OCH_2Ar), 112.4 (d), 114.4 (d), 126.0 (d), 127.1 (2×d), 127.6 (d), 128.3 (2×d), 131.4 (s), 136.9 (s), 137.0 (s), 156.7 (s, C-3), 171.3 (s, C=O) and 178.2 (s, C=O); MS *m/z* (FAB+) 446.3 [97, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 446.2691, $C_{29}H_{36}NO_3$ requires 446.2695. Found: C, 77.80; H, 7.89; N, 3.13. $C_{29}H_{35}NO_3$ requires: C, 78.17; H, 7.92; N, 3.14%.

3-Benzyloxy-*N*-pentyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (10)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with pentyl bromide (318 μL, 2.57 mmol) was complete within 5 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **10** as a white residue (550 mg, 93%). An analytical sample was recrystallised from EtOH to give colourless needles: mp 104-107°C; IR (KBr) 3100-3000 (arom CH), 2960-2870 (aliph CH), 1720 (C=O), 1660 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.89 (3H, t, J = 7.2 Hz, C-5′-H_3), 1.16 (3H, s, C-18-H_3), 1.20-2.98 (17H, m), 2.83-2.89 (2H, m, C-6-H_2), 3.66-3.82 (2H, m, N-CH_2), 5.03 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.80 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.21 (1H, d, J = 8.6 Hz, C-1-H) and 7.30-7.44 (5H, m, C_6H_5); δ_C

($CDCl_3$, 100.4 MHz) 14.1 (q, C-5′), 16.6 (q, C-18), 22.5 (t), 25.6 (t), 25.8 (t), 27.7 (t), 29.2 (t), 29.7 (t), 33.7 (t), 33.8 (t), 38.7 (d), 40.1 (t, C-1′), 40.3 (d), 41.5 (s, C-13), 42.5 (d), 70.0 (t, OCH_2Ar), 112.5 (d), 114.5 (d), 126.1 (d), 127.3 (2xd), 127.8 (d), 128.4 (2xd), 131.5 (s), 137.0 (s), 137.2 (s), 156.8 (s, C-3), 171.4 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 460.2 [78, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 460.2845, $C_{30}H_{38}NO_3$ requires 460.2852. Found: C, 78.20; H, 8.08; N, 3.01. $C_{30}H_{37}NO_3$ requires: C, 78.40; H, 8.11; N, 3.05%.

3-Benzyloxy-*N*-hexyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (11)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with hexyl bromide (360 µL, 2.57 mmol) was complete within 1.5 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **11** as a white residue (575 mg, 94%). An analytical sample was recrystallised from EtOH to give white needles: mp 108-111°C; IR (KBr) 2960-2860 (aliph CH), 1720 (C=O), 1665 (C=O), 1615-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.87 (3H, t, J = 6.6 Hz, C-6′-H_3), 1.16 (3H, s, C-18-H_3), 1.28-2.98 (19H, m), 2.84-2.89 (2H, m, C-6-H_2), 3.74 (2H, m, N-CH_2), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.81 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.22 (1H, d, J = 8.6 Hz, C-1-H) and 7.29-7.44 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 14.1 (q, C-6′), 16.6 (q, C-18), 22.6 (t), 25.6 (t), 25.8 (t), 26.7 (t), 28.0 (t), 29.8 (t), 31.6 (t), 33.7 (t), 33.8 (t), 38.7 (d), 40.1 (t, C-1′), 40.4 (d), 41.5 (s, C-13), 42.5 (d), 70.0 (t, OCH_2Ar), 112.6 (d), 114.5 (d), 126.1 (d), 127.3 (2×d), 127.8 (d), 128.4 (2×d), 131.6 (s), 137.0 (s), 137.2 (s), 156.8 (s, C-3), 171.4 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 474.3 [68, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 474.2988, $C_{31}H_{40}NO_3$ requires 474.3008. Found: C, 78.10; H, 8.16; N, 2.98. $C_{31}H_{39}NO_3$ requires: C, 78.61; H, 8.30; N, 2.96%.

3-Benzyloxy-*N*-(4′-bromobutyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (12)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with 1,4-dibromobutane (310 µL, 2.57 mmol) was complete within 1.5 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **12** as a white residue (569 mg, 84%). An analytical

sample was recrystallised from EtOH to give white crystals: mp 113-116°C; IR (KBr) 2935-2860 (aliph CH), 1720 (C=O), 1670 (C=O), 1605-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.17 (3H, s, C-18-H_3), 1.30-3.00 (15H, m), 2.84-2.90 (2H, m, C-6-H_2), 3.42 (2H, t, *J* = 6.8 Hz, C-4′-H_2), 3.79 (2H, m, N-CH_2), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, *J* = 2.7 Hz, C-4-H), 6.81 (1H, dd, *J* = 8.6 Hz, *J* = 2.7 Hz, C-2-H), 7.21 (1H, d, *J* = 8.6 Hz, C-1-H) and 7.30-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 17.0 (q, C-18), 25.9 (t), 26.1 (t), 27.1 (t), 30.1 (t), 30.5 (t), 33.5 (t), 33.9 (t), 34.1 (t), 39.0 (d), 39.3 (t, C-1′), 40.6 (d), 41.8 (s, C-13), 42.8 (d), 70.3 (t, OCH_2Ar), 112.9 (d), 114.8 (d), 126.4 (d), 127.6 (2×d), 128.1 (d), 128.8 (2×d), 131.8 (s), 137.3 (s), 137.5 (s), 157.1 (s, C-3), 171.8 (s, C=O) and 178.8 (s, C=O); MS *m/z* (FAB+) 524.1 [42, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 526.1759, $C_{29}H_{35}{}^{81}BrNO_3$ requires 526.1780 and 524.1738, $C_{29}H_{35}BrNO_3$ requires 524.1800. Found: C, 66.30; H, 6.51; N, 2.56. $C_{29}H_{34}BrNO_3$ requires: C, 66.41; H, 6.53; N, 2.67%.

3-Benzyloxy-*N*-cyclopropylmethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (13)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with bromomethyl-cyclopropane (246 μL, 2.57 mmol) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **13** as a white residue (536 mg, 94%). An analytical sample was recrystallised from EtOH to give white needles: mp 96-99°C; IR (KBr) 2920-2860 (aliph CH), 1720 (C=O), 1670 (C=O), 1610-1495 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.29-0.34 (2H, m, C-3′-H_2), 0.40-0.45 (2H, m, C-4′-H_2), 1.15 (1H, m, C-2′-H), 1.18 (3H, s, C-18-H_3), 1.25-3.01 (11H, m), 2.85-2.90 (2H, m, C-6-H_2), 3.67 (2H, m, N-CH_2), 5.04 (2H, s, OCH_2Ar), 6.73 (1H, d, *J* = 2.5 Hz, C-4-H), 6.81 (1H, dd, *J* = 8.6 Hz, *J* = 2.5 Hz, C-2-H), 7.22 (1H, d, *J* = 8.6 Hz, C-1-H) and 7.29-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 3.9 (t, C-3′), 4.0 (t, C-4′), 10.5 (d, C-2′), 17.0 (q, C-18), 26.0 (t), 26.1 (t), 30.1 (t), 34.0 (t), 34.1 (t), 39.1 (d), 40.6 (d), 41.9 (s, C-13), 42.9 (d), 44.6 (t, C-1′), 70.3 (t, OCH_2Ar), 112.9 (d), 114.9 (d), 126.4 (d), 127.6 (2×d), 128.1 (d), 128.7 (2×d), 131.9 (s), 137.4 (s), 137.5 (s), 157.2 (s, C-3), 172.0 (s, C=O) and 179.0 (s, C=O); MS *m/z* (FAB+) 887.3 [58, $(2M+H)^+$], 444.1 [98, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 444.2525, $C_{29}H_{34}NO_3$ requires

444.2539. Found: C, 78.30; H, 7.47; N, 3.18. $C_{29}H_{33}NO_3$ requires: C, 78.52; H, 7.50; N, 3.16%.

3-Benzyloxy-*N*-(4″-*tert*-butyl-benzyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (14)

Following method 1, **5** (500 mg, 1.28 mmol) was treated with NaH (62 mg, 1.54 mmol) and the subsequent reaction with 1-bromomethyl-4-*tert*-butyl-benzene (472 μL, 2.57 mmol) was complete within 30 minutes. The crude product was purified by flash chromatography ($CHCl_3$) to give **14** as a white residue (667 mg, 97%): mp 199-200°C; IR (KBr) 2965-2870 (aliph CH), 1720 (C=O), 1670 (C=O), 1605-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.16 (3H, s, C-18-H_3), 1.28 (9H, s, $C(CH_3)_3$), 1.30-3.01 (11H, m), 2.84-2.90 (2H, m, C-6-H_2), 4.88 (1H, d, J_{BA} = 13.7 Hz, N-CH_ACH_B), 4.94 (1H, d, J_{AB} = 14.1 Hz, N-CH_ACH_B), 5.03 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.80 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.21 (1H, d, J = 8.6 Hz, C-1-H) and 7.24-7.44 (9H, m, C_6H_5, C-2″-H, C-3″-H, C-5″-H and C-6″-H); δ_C ($CDCl_3$, 100 .4 MHz) 16.6 (q, C-18), 25.6 (t), 25.8 (t), 29.7 (t), 31.4 (3×q, C(CH_3)$_3$), 33.7 (t), 33.8 (t), 34.5 (s, $C(CH_3)_3$), 38.6 (d), 40.1 (d), 41.5 (s, C-13), 42.5 (d), 42.9 (t, C-1′), 69.9 (t, OCH_2Ar), 112.5 (d), 114.5 (d), 125.2 (2×d), 126.1 (d), 127.3 (2×d), 127.8 (d), 128.0 (2×d), 128.4 (2×d), 131.5 (s), 134.2 (s), 137.0 (s), 137.2 (s), 149.9 (s), 156.8 (s, C-3), 171.4 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 1071.5 [32, $(2M+H)^+$], 536.2 [80, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; MS *m/z* (FAB-) 534.3 [72, $(M-H)^-$], 195.0 [100], 276.0 [100]; Acc MS *m/z* (FAB+) 536.3150, $C_{36}H_{42}NO_3$ requires 536.3165.

3-Benzyloxy-*N*-(1″-pyridin-3″-ylmethyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (15)

3-Picolylamine (4.71 mL, 46.24 mmol) and **4** (600 mg, 1.47 mmol) were heated at 180°C under an atmosphere of N_2 for 2 hours. After cooling down, the resulting orange mixture was poured into H_2O (300 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×70 mL), washed with H_2O (2×50 mL), brine (2×50 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was recrystallised from EtOH to give **15** as light yellow crystals (543

mg, 77%). An analytical sample was further recrystallised from EtOH to give colourless needles: mp 170-172°C; IR (KBr) 2925-2870 (aliph CH), 1720 (C=O), 1670 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.14 (3H, s, C-18-H_3), 1.28-3.04 (11H, m), 2.84-2.88 (2H, m, C-6-H_2), 4.92 (1H, d, J_{BA}= 13.7 Hz, N-CH_AH$_B$), 4.98 (1H, d, J_{AB} = 14.1 Hz, N-CH$_A$$H_B$), 5.03 (2H, s, OCH_2Ar), 6.71 (1H, d, J = 2.7 Hz, C-4-H), 6.79 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.17-7.45 (7H, m, C_6H_5, C-1-H and C-5″-H), 7.69 (1H, dt, J = 7.8 Hz, J = 1.8 Hz, C-4″-H), 8.50 (1H, dd, J = 5.1 Hz, J = 1.8 Hz, C-6″-H) and 8.63 (1H, d, J = 1.8 Hz, C-2″-H); δ_C ($CDCl_3$, 100.4 MHz) 16.5 (q, C-18), 25.5 (t), 25.8 (t), 29.7 (t), 33.6 (t), 33.7 (t), 38.5 (d), 40.1 (d), 40.8 (t, C-1′), 41.6 (s, C-13), 42.4 (d), 69.9 (t, OCH_2Ar), 112.5 (d), 114.5 (d), 123.2 (d), 126.1 (d), 127.3 (2×d), 127.7 (d), 128.4 (2×d), 131.3 (s), 132.8 (s), 136.4 (d), 137.1 (2×s), 148.6 (d), 150.0 (d), 156.8 (s, C-3), 171.4 (s, C=O) and 178.3 (s, C=O); MS *m/z* (FAB+) 481.3 [100, $(M+H)^+$], 91.1 [47, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 481.2504, $C_{31}H_{33}N_2O_3$ requires 481.2491. Found: C, 77.00; H, 6.75; N, 5.73. $C_{32}H_{32}N_2O_3$ requires: C, 77.47; H, 6.71; N, 5.83%.

3-Benzyloxy-*N*-benzyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (16)

Benzylamine (6.25 mL, 57.22 mmol) and **4** (500 mg, 1.22 mmol) were heated at 180°C under an atmosphere of N_2 for 3 hours. After cooling down, the resulting brown mixture was poured into H_2O (250 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (50 mL), washed with H_2O (2×25 mL), brine (3×25 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography ($CHCl_3$/hexane, 8:2) to give **16** as an off-white powder (385 mg, 65%). An analytical sample was recrystallised from MeOH to give colourless needles: mp 144-146°C; IR (KBr) 3100 (arom CH), 2940-2850 (aliph CH), 1720 (C=O), 1670 (C=O), 1615-1560 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.15 (3H, s, C-18-H_3), 1.25-3.01 (11H, m), 2.84-2.89 (2H, m, C-6-H_2), 4.91 (1H, d, J_{BA} = 13.7 Hz, N-CH_AH$_B$), 4.98 (1H, d, J_{AB} = 13.7 Hz, N-CH$_A$$H_B$), 5.03 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.80 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.21 (1H, d, J = 8.4 Hz, C-1-H) and 7.24-7.43 (10H, m, 2×C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 16.5 (q, C-18), 25.6 (t), 25.8 (t), 29.7 (t), 33.7 (2×t), 38.6 (d), 40.2 (d), 41.5 (s, C-13), 42.5 (d), 43.2 (t, C-1′), 69.9 (t, OCH_2Ar),

112.5 (d), 114.5 (d), 126.1 (d), 127.2 (d), 127.3 (2×d), 127.8 (d), 128.3 (2×d), 128.4 (4×d), 131.5 (s), 137.0 (s), 137.2 (2×s), 156.8 (s, C-3), 171.4 (s, C=O) and 178.3 (s, C=O); MS *m/z* (FAB+) 480.2 [52, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 480.2522, $C_{32}H_{34}NO_3$ requires 480.2539. Found: C, 80.10; H, 6.91; N, 2.94. $C_{32}H_{33}NO_3$ requires: C, 80.14; H, 6.94; N, 2.92%.

3-Hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (17)

Following method 2, a suspension of **5** (350 mg, 899 μmol) and Pd-C (10%, 100 mg) in MeOH/THF 1:1 (50 mL) was hydrogenated for 5 hours to give **17** as a white solid (246 mg, 91%). An analytical sample was recrystallised from $CHCl_3$/Hexane (2:1) to give white crystals: mp 297-300°C [lit.[160] (MeOH) 294-296°C]; IR (KBr) 3410 (OH), 3180-3085 (arom CH), 2955-2870 (aliph CH), 1715 (C=O), 1680 (C=O), 1615-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.09 (3H, s, C-18-H_3), 1.15-2.66 (11H, m), 2.69-2.73 (2H, m, C-6-H_2), 6.44 (1H, d, J = 2.7 Hz, C-4-H), 6.52 (1H, dd, J = 8.5 Hz, J = 2.7 Hz, C-2-H), 7.07 (1H, d, J = 8.5 Hz, C-1-H), 9.05 (1H, s, exchanged with D_2O, OH) and 10.63 (1H, s, exchanged with D_2O, NH); δ_C (DMSO-d_6, 100.4 MHz) 16.2 (q, C-18), 25.2 (t), 25.4 (t), 29.2 (t), 32.4 (t), 32.8 (t), 38.0 (d), 40.4 (d), 40.5 (s, C-13), 41.4 (d), 112.7 (d), 114.5 (d), 125.9 (d), 129.5 (s), 136.7 (s), 154.8 (s, C-3), 172.2 (s, C=O) and 179.0 (s, C=O); MS *m/z* (FAB+) 300.0 [100, $(M+H)^+$], 133.0 [29], 111.1 [36], 97.1 [60]; MS *m/z* (FAB-) 451.3 [58, $(M-H+NBA)^-$], 298.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 300.1585, $C_{18}H_{22}NO_3$ requires 300.1600. HPLC (MeOH/H_2O, 60:40; λ_{max} = 279.3 nm) Rt = 3.06 min, 100%. Found: C, 61.80; H, 5.85; N, 3.86. $C_{18}H_{21}NO_3.(CHCl_3)_{1/2}$ requires: C, 61.88; H, 6.04; N, 3.90%.

3-Hydroxy-*N*-methyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (18)

Following method 2, a suspension of **6** (400 mg, 992 μmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 2 hours to give **18** as a white solid (253 mg, 81%). An analytical sample was recrystallised from EtOAc to give white crystals: mp 328-330°C; IR (KBr) 3460 (OH), 2940-2860 (aliph CH), 1715 (C=O), 1655 (C=O), 1610-1510 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.09 (3H, s, C-18-H_3), 1.19-2.97 (11H, m), 2.68-2.73 (2H, m, C-6-H_2), 2.98 (3H, s, N-CH_3), 6.44

(1H, d, J = 2.3 Hz, C-4-H), 6.52 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H), 7.06 (1H, d, J = 8.5 Hz, C-1-H) and 9.05 (1H, s, exchanged with D_2O, OH); δ_C (DMSO-d_6, 100.4 MHz) 16.3 (q, C-18), 25.2 (t), 26.4 (q, C-1′), 29.1 (t), 32.8 (t), 33.5 (2×t), 37.8 (d), ~39 (s, under solvent peaks), 40.9 (d), 41.9 (d), 112.7 (d), 114.5 (d), 125.8 (d), 129.4 (s), 136.7 (s), 154.8 (s, C-3), 171.3 (s, C=O) and 178.2 (s, C=O); MS *m/z* (FAB+) 314.1 [78, $(M+H)^+$], 97.1 [100]; Acc MS *m/z* (FAB+) 314.1749, $C_{19}H_{24}NO_3$ requires 314.1756. HPLC (MeOH/H_2O, 70:30; λ_{max} = 279.3 nm) Rt = 3.24 min, 100%. Found: C, 72.60; H, 7.16; N, 4.35. $C_{19}H_{23}NO_3$ requires: C, 72.82; H, 7.40; N, 4.47%.

3-Hydroxy-*N*-ethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (19)

Following method 2, a suspension of **7** (470 mg, 1.13 mmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 4.5 hours to give **19** as a white solid (183 mg, 50%). This product was washed with acetone to give a white precipitate (121 mg, 33%): mp 306-308°C; IR (KBr) 3450 (OH), 2915-2860 (aliph CH), 1715 (C=O), 1655 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.11 (3H, t, J = 7.0 Hz, C-2′-H_3), 1.16 (3H, s, C-18-H_3), 1.22-2.98 (11H, m), 2.81-2.87 (2H, m, C-6-H_2), 3.82 (2H, m, N-CH_2), 4.62 (1H, s, exchanged with D_2O, OH), 6.57 (1H, d, J = 2.7 Hz, C-4-H), 6.66 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H) and 7.17 (1H, d, J = 8.6 Hz, C-1-H); MS *m/z* (FAB+) 328.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 328.1906, $C_{20}H_{26}NO_3$ requires 328.1913. Found: C, 72.90; H, 7.68; N, 4.09. $C_{20}H_{25}NO_3$ requires: C, 73.37; H, 7.70; N, 4.28%.

3-Hydroxy-*N*-propyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (20)

Following method 2, a suspension of **8** (400 mg, 927 μmol) and Pd-C (10%, 100 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 3 hours to give **20** as a white solid (256 mg, 81%). An analytical sample was recrystallised from MeOH to give colourless crystals: mp 183-186°C; IR (KBr) 3445 (OH), 3050 (arom CH), 2940-2860 (aliph CH), 1725 (C=O), 1655 (C=O), 1585-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.90 (3H, t, J = 7.4 Hz, C-3′-H_3), 1.17 (3H, s, C-18-H_3), 1.30-2.98 (13H, m), 2.82-2.86 (2H, m, C-6-H_2), 3.64-3.80 (2H, m, N-CH_2), 4.73 (1H, s, exchanged with D_2O, OH), 6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.66 (1H, dd, $J_{C\text{-}1\text{-}H,C\text{-}2\text{-}H}$ = 8.6 Hz, J = 2.7 Hz, C-2-H) and 7.17 (1H, d, J = 8.6 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 11.4 (q, C-3′), 16.6 (q, C-18), 21.3 (t), 25.6 (d), 25.8 (t), 29.6 (t), 33.6 (t), 33.7

(t), 38.7 (d), 40.3 (d), 41.5 (s, C-13), 41.6 (t, C-1′), 42.5 (d), 113.0 (d), 115.0 (d), 126.3 (d), 131.1 (s), 137.4 (s), 153.7 (s, C-3), 171.8 (s, C=O) and 178.6 (s, C=O); MS *m/z* (FAB+) 342.3 [100, $(M+H)^+$], 97.2 [45]; MS *m/z* (FAB-) 494.4 [43, $(M+NBA)^-$], 340.3 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 342.2076, $C_{21}H_{28}NO_3$ requires 342.2069. HPLC ($MeOH/H_2O$, 70:30; λ_{max} = 279.3 nm) Rt = 6.55 min, 100%. Found: C, 73.90; H, 7.98; N, 4.20. $C_{21}H_{27}NO_3$ requires: C, 73.87; H, 7.97; N, 4.10%.

3-Hydroxy-*N*-butyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (21)

Following method 2, a suspension of **9** (480 mg, 1.08 mmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 2 hours to give **21** as a white solid (361 mg, 94%). This product was recrystallised from MeOH to give colourless needles (242 mg, 63%): mp 212-214°C [lit.[160] (MeOH) 210-212°C]; IR (KBr) 3445 (OH), 2940-2870 (aliph CH), 1715 (C=O), 1655 (C=O), 1585-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, t, *J* = 7.2 Hz, C-4′-H_3), 1.16 (3H, s, C-18-H_3), 1.26-2.99 (15H, m), 2.81-2.88 (2H, m, C-6-H_2), 3.75 (2H, m, N-CH_2), 4.75 (1H, s, exchanged with D_2O, OH), 6.58 (1H, d, *J* = 2.7 Hz, C-4-H), 6.66 (1H, dd, *J* = 8.6 Hz, *J* = 2.7 Hz, C-2-H) and 7.17 (1H, d, *J* = 8.6 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 13.8 (q, C-4′), 16.5 (q, C-18), 20.1 (t), 25.5 (t), 25.7 (t), 29.4 (t), 30.0 (t), 33.5 (t), 33.7 (t), 38.6 (d), 39.8 (t, C-1′), 40.2 (d), 41.4 (s, C-13), 42.4 (d), 112.9 (d), 114.9 (d), 126.2 (d), 131.1 (s), 137.3 (s), 153.5 (s, C-3), 171.5 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 356.2 [100, $(M+H)^+$]; MS *m/z* (FAB-) 508.2 [35, $(M+NBA)^-$], 354.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 356.2225, $C_{22}H_{30}NO_3$ requires 356.2226. Found: C, 74.20; H, 8.21; N, 3.88. $C_{22}H_{29}NO_3$ requires: C, 74.33; H, 8.22; N, 3.94%.

3-Hydroxy-*N*-pentyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (22)

Following method 2, a suspension of **10** (520 mg, 1.13 mmol) and Pd-C (10%, 100 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 3 hours to give **22** as a white solid (347 mg, 83%). An analytical sample was recrystallised from MeOH to give white crystals: mp 181-184°C; IR (KBr) 3445 (OH), 2955-2870 (aliph CH), 1715 (C=O), 1660 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.89 (3H, t, *J* = 7.4 Hz, C-5′-H_3), 1.16 (3H, s, C-18-H_3), 1.20-2.98 (17H, m), 2.81-2.86 (2H, m, C-6-H_2), 3.65-3.82 (2H, m, N-CH_2), 4.77-4.79 (1H, m, exchanged with D_2O, OH),

6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.65 (1H, dd, J = 8.2 Hz, J = 2.7 Hz, C-2-H) and 7.17 (1H, d, J = 8.2 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 14.1 (q, C-5′), 16.6 (q, C-18), 22.5 (t), 25.6 (t), 25.8 (t), 27.7 (t), 29.1 (t), 29.7 (t), 33.7 (t), 33.8 (t), 38.7 (d), 40.2 (t, C-1′), 40.3 (d), 41.5 (s, C-13), 42.5 (d), 113.0 (d), 115.0 (d), 126.3 (d), 131.2 (s), 137.4 (s), 153.6 (s, C-3), 171.7 (s, C=O) and 178.5 (s, C=O); MS *m/z* (FAB+) 739.1 [50, $(2M+H)^+$], 370.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 522.4 [30, $(M+NBA)^-$], 368.3 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 370.2394, $C_{23}H_{32}NO_3$ requires 370.2382. HPLC ($MeOH/H_2O$, 80:20; λ_{max} = 279.3 nm) Rt = 5.42 min, 100%. Found: C, 74.90; H, 8.38; N, 3.73. $C_{23}H_{31}NO_3$ requires: C, 74.96; H, 8.46; N, 3.79%.

3-Hydroxy-*N*-hexyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (23)

Following method 2, a suspension of **11** (540 mg, 1.14 mmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (45 mL) was hydrogenated for 3 hours to give **27** as a white solid (384 mg, 88%). This product was recrystallised from MeOH to give white crystals (263 mg, 60%): mp 157-159°C; IR (KBr) 3435 (OH), 2930-2865 (aliph CH), 1715 (C=O), 1660 (C=O), 1585-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.87 (3H, t, J = 6.8 Hz, C-6′-H_3), 1.16 (3H, s, C-18-H_3), 1.23-2.98 (19H, m), 2.81-2.87 (2H, m, C-6-H_2), 3.74 (2H, m, N-CH_2), 4.68 (1H, s, exchanged with D_2O, OH), 6.58 (1H, d, J = 2.5 Hz, C-4-H), 6.66 (1H, dd, J = 8.4 Hz, J = 2.5 Hz, C-2-H) and 7.17 (1H, d, J = 8.4 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 14.0 (q, C-6′), 16.5 (q, C-18), 22.5 (t), 25.5 (t), 25.6 (t), 26.5 (t), 27.8 (t), 29.4 (t), 31.4 (t), 33.5 (t), 33.6 (t), 38.5 (d), 40.1 (d and t, C-1′), 41.3 (s, C-13), 42.3 (d), 112.8 (d), 114.8 (d), 126.2 (d), 131.0 (s), 137.2 (s), 153.5 (s, C-3), 171.6 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 767.6 [48, $(2M+H)^+$], 384.3 [100, $(M+H)^+$]; MS *m/z* (FAB-) 382.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 384.2535, $C_{24}H_{34}NO_3$ requires 384.2539. Found: C, 75.40; H, 8.65; N, 3.71. $C_{24}H_{33}NO_3$ requires: C, 75.16; H, 8.67; N, 3.65%.

3-Hydroxy-*N*-(4′-bromobutyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (24)

Following method 2, a suspension of **12** (210 mg, 381 μmol) and Pd-C (10%, 100 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 2 hours to give **24** as a white solid (146 mg, 84%). This product was recrystallised from MeOH to give white crystals (98 mg, 57%): mp 165-167°C; IR (KBr) 3450 (OH), 2910-2860 (aliph CH), 1715 (C=O), 1660 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.18

(3H, s, C-18-H_3), 1.29-3.01 (15H, m), 2.82-2.88 (2H, m, C-6-H_2), 3.42 (2H, t, J = 6.6 Hz, C-4′-H_2), 3.72-3.87 (2H, m, N-CH_2), 4.63 (1H, s, exchanged with D_2O, OH), 6.58 (1H, d, J = 2.3 Hz, C-4-H), 6.66 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H) and 7.17 (1H, d, J = 8.5 Hz, C-1-H); δ_C (DMSO-d_6, 100.4 MHz) 17.0 (q, C-18), 25.9 (t), 26.1 (t), 27.1 (t), 29.9 (t), 30.5 (t), 33.5 (t), 33.9 (t), 34.1 (t), 39.0 (d), 39.4 (t, C-1′), 40.6 (d), 41.9 (s, C-13), 42.8 (d), 113.3 (d), 115.3 (d), 126.6 (d), 131.5 (s), 137.7 (s), 153.9 (s, C-3), 171.9 (s, C=O) and 178.8 (s, C=O); MS *m/z* (FAB+) 869.2 [64], 587.1 [46, $(M+H+NBA)^+$], 434.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 436.1287, $C_{22}H_{29}{}^{81}BrNO_3$ requires 436.1310 and 434.1282, $C_{22}H_{29}BrNO_3$ requires 434.1331. HPLC (MeOH/H_2O, 70:30; λ_{max} = 279.3 nm) Rt = 7.73 min, 98%. Found: C, 61.30; H, 6.60; N, 3.17. $C_{22}H_{28}BrNO_3$ requires: C, 60.83; H, 6.50; N, 3.22%.

3-Hydroxy-*N*-cyclopropylmethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (25)

Following method 2, a suspension of **13** (500 mg, 1.13 mmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (45 mL) was hydrogenated for 2.5 hours to give **25** as a white solid (356 mg, 89%). This product was recrystallised from MeOH to give colourless crystals (254 mg, 64%): mp 238-240°C; IR (KBr) 3440 (OH), 2940-2865 (aliph CH), 1715 (C=O), 1655 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.29-0.34 (2H, m, C-3′-H_2), 0.40-0.45 (2H, m, C-4′-H_2), 1.13 (1H, m, C-2′-H), 1.19 (3H, s, C-18-H_3), 1.30-3.02 (11H, m), 2.82-2.89 (2H, m, C-6-H_2), 3.66 (2H, m, N-CH_2), 4.70 (1H, s, exchanged with D_2O, OH), 6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.66 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H) and 7.17 (1H, d, J = 8.4 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 3.9 (t, C-3′), 4.0 (t, C-4′), 10.5 (d, C-2′), 16.9 (q, C-18), 26.0 (t), 26.1 (t), 29.9 (t), 31.1 (t), 34.0 (t), 39.0 (d), 40.6 (d), 41.9 (s, C-13), 42.8 (d), 44.6 (t, C-1′), 113.3 (d), 115.3 (d), 126.6 (d), 131.6 (s), 137.7 (s), 154.0 (s, C-3), 172.2 (s, C=O) and 179.1 (s, C=O); MS *m/z* (FAB+) 707.3 [29, $(2M+H)^+$], 507.1 [72, $(M+H+NBA)^+$], 354.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 505.2 [32, $(M-H+NBA)^-$], 352.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 354.2069, $C_{22}H_{28}NO_3$ requires 354.2069. Found: C, 74.70; H, 7.55; N, 3.88. $C_{22}H_{27}NO_3$ requires: C, 74.76; H, 7.70; N, 3.96%.

3-Hydroxy-*N*-(4″-*tert*-butyl-benzyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (26)

Following method 2, a suspension of **14** (620 mg, 1.16 mmol) and Pd-C (10%, 200 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 5 hours to give **26** as an off-white solid (550 mg). This product was recrystallised from MeOH to give white flaky crystals (448 mg, 87%): mp 128-130°C; IR (KBr) 3415 (OH), 2955-2870 (aliph CH), 1725 (C=O), 1655 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.16 (3H, s, C-18-H_3), 1.28 (9H, s, $C(CH_3)_3$), 1.30-3.02 (15H, m), 2.81-2.87 (2H, m, C-6-H_2), 4.77 (1H, s, exchanged with D_2O, OH), 4.88 (1H, d, J_{BA} = 14.0 Hz, N-C$H_A$$H_B$), 4.95 (1H, d, J_{AB} = 14.0 Hz, N-C$H_A$$H_B$), 6.57 (1H, d, J = 2.7 Hz, C-4-H), 6.65 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.16 (1H, d, J = 8.4 Hz, C-1-H) and 7.24-7.32 (4H, m, C-2″-H, C-3″-H, C-5″-H and C-6″-H); δ_C ($CDCl_3$, 100.4 MHz) 16.9 (q, C-18), 25.9 (t), 26.1 (t), 29.9 (t), 31.7 (3×q, $C(CH_3)_3$), 34.0 (t), 34.1 (t), 34.9 (s, $C(CH_3)_3$), 39.0 (d), 40.4 (d), 41.9 (s, C-13), 42.8 (d), 43.3 (t, C-1′), 113.3 (d), 115.3 (d), 125.5 (2×d), 126.6 (d), 128.3 (2×d), 131.4 (s), 134.4 (s), 137.7 (s), 150.3 (s), 153.9 (s, C-3), 172.0 (s, C=O) and 178.8 (s, C=O); MS *m/z* (FAB+) 891.4 [80, $(2M+H)^+$], 599.2 [35, $(M+H+NBA)^+$], 446.2 [100, $(M+H)^+$]; MS *m/z* (FAB-) 889.5 [42, $(2M-H)^-$], 751.4 [87, $(M+2NBA)^-$], 598.3 [30, $(M+NBA)^-$], 444.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 446.2690, $C_{29}H_{36}NO_3$ requires 446.2695. Found: C, 77.40; H, 7.67; N, 3.19. $C_{29}H_{35}NO_3.(H_2O)_{1/4}$ requires: C, 77.39; H, 7.95; N, 3.11%.

3-Hydroxy-*N*-(1″-pyridin-3″-ylmethyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (27)

Following method 2, a suspension of **15** (190 mg, 395 μmol) and Pd-C (10%, 100 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 20 hours to give **27** as an off-white solid (141 mg, 91%). An analytical sample was triturated with EtOAc to give a white powder: mp 199-203°C; IR (KBr) 3380 (OH), 2940-2865 (aliph CH), 1720 (C=O), 1670 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.11 (3H, s, C-18-H_3), 1.14-2.94 (11H, m), 2.67-2.75 (2H, m, C-6-H_2), 4.82 (1H, d, J_{BA} = 14.8 Hz, N-C$H_A$$H_B$), 4.87 (1H, d, J_{AB} = 14.8 Hz, N-C$H_A$$H_B$), 6.44 (1H, d, J = 2.3 Hz, C-4-H), 6.52 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H), 7.07 (1H, d, J = 8.5 Hz, C-1-H), 7.33 (1H, dd, J = 7.8 Hz, J = 4.7 Hz, C-5″-H), 7.57-7.61 (1H, m, C-4″-H), 8.42-

8.47 (2H, m, C-2″-H and C-6″-H) and 9.05 (1H, s, exchanged with D_2O, OH); MS *m/z* (FAB+) 391.2 [88, $(M+H)^+$], 156.1 [40], 135.1 [46], 119.1 [48], 95.1 [70]; MS *m/z* (FAB-) 542.3 [50, $(M-H+NBA)^-$], 389.3 [100, $(M-H)^-$], 276.1 [43], 258.1 [37], 195.1 [42], 124.1 [34]; Acc MS *m/z* (FAB+) 391.2019, $C_{24}H_{27}N_2O_3$ requires 391.2022.

3-Hydroxy-*N*-benzyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (28)

Following method 2, a suspension of **16** (230 mg, 479 μmol) and Pd-C (10%, 100 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 2 hours to give **28** as a white solid (170 mg, 91%). This product was triturated with boiling MeOH to give a white precipitate (122 mg, 65%): mp 298-301°C; IR (KBr) 3430 (OH), 2950-2890 (aliph CH), 1720 (C=O), 1655 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.12 (3H, s, C-18-H_3), 1.18-2.92 (11H, m), 2.68-2.75 (2H, m, C-6-H_2), 4.79 (1H, d, J_{BA} = 14.8 Hz, N-C$H_A$$H_B$), 4.85 (1H, d, J_{AB} = 14.8 Hz, N-C$H_A$$H_B$), 6.45 (1H, d, J = 2.3 Hz, C-4-H), 6.53 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H), 7.07 (1H, d, J = 8.5 Hz, C-1-H), 7.17-7.32 (5H, m, C_6H_5) and 9.05 (1H, s, exchanged with D_2O, OH); δ_C (DMSO-d_6, 100.4 MHz) 16.3 (q, C-18), 25.2 (2×t), 29.1 (t), 32.9 (t), 33.5 (t), 38.0 (d), ~39 (d, under solvent peaks), 41.0 (s, C-13), 41.7 (d), 42.3 (t, C-1′), 112.7 (d), 114.5 (d), 125.8 (d), 126.6 (d), 126.8 (2×d), 128.1 (2×d), 129.4 (s), 136.7 (s), 137.4 (s), 154.8 (s, C-3), 172.2 (s, C=O) and 178.0 (s, C=O); MS *m/z* (FAB+) 390.3 [30, $(M+H)^+$], 133.2 [43], 111.2 [57], 97.2 [100]; Acc MS *m/z* (FAB+) 390.2062, $C_{25}H_{28}NO_3$ requires 390.2069. Found: C, 75.60; H, 7.01; N, 3.34. $C_{25}H_{27}NO_3.(H_2O)_{1/2}$ requires: C, 75.35; H, 7.08; N, 3.51%.

3-*O*-*tert*-butyl-dimethylsilyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (29)

Imidazole (256 mg, 3.74 mmol) and *tert*-butyl-dimethylsilyl chloride (546 mg, 3.63 mmol) were added to a stirred solution of **17** (350 mg, 1.17 mmol) in anhydrous DMF (20 mL) at room temperature under an atmosphere of N_2. The resulting mixture was stirred for 4 hours, after which it was poured into H_2O (150 mL). The organics were extracted with EtOAc (150 mL), washed with H_2O (4×80 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a white solid. The crude product was recrystallised from EtOH/H_2O to give **29** as white crystals (376 mg, 78%): mp 261-264°C; IR (KBr) 3210 (NH), 3090 (arom CH), 2950-2860 (aliph CH),

1730 (C=O), 1680 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.19 (6H, s, $Si(CH_3)_2$), 0.97 (9H, s, $C(CH_3)_3$), 1.23 (3H, s, C-18-H_3), 1.31-2.96 (11H, m), 2.80-2.87 (2H, m, C-6-H_2), 6.57 (1H, d, J = 2.5 Hz, C-4-H), 6.64 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H), 7.13 (1H, d, J = 8.6 Hz, C-1-H) and 7.72 (1H, s, exchanged with D_2O, NH); δ_C ($CDCl_3$, 100.4 MHz) -4.2 (2×q, $Si(CH_3)_2$), 16.5 (q, C-18), 18.3 (s, $C(CH_3)_3$), 25.4 (t), 25.8 (3×q, $C(CH_3)_3$), 26.1 (t), 29.5 (t), 32.8 (t), 32.9 (t), 38.5 (d), 41.2 (s, C-13), 41.6 (d), 42.7 (d), 117.5 (d), 119.7 (d), 126.0 (d), 131.5 (s), 136.9 (s), 153.5 (s, C-3), 171.6 (s, C=O) and 178.4 (s, C=O); MS *m/z* (FAB+) 827.6 [50, $(2M+H)^+$], 414.2 [100, $(M+H)^+$], 356.2 [45, $(M-C(CH_3)_3)^+$], 72.9 [50]; MS *m/z* (FAB-) 412.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 414.2453, $C_{24}H_{36}NO_5Si$ requires 414.2464. Found: C, 69.60; H, 8.46; N, 3.40. $C_{24}H_{35}NO_5Si$ requires: C, 69.69; H, 8.53; N, 3.39%.

3-*O*-*tert*-butyl-dimethylsilyl-*N*-allyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (30)

Following method 1, **29** (300 mg, 725 μmol) was treated with NaH (35 mg, 870 μmol) and the subsequent reaction with allyl bromide (126 μL, 1.45 mmol) was complete within 7 hours. The crude product was purified by flash chromatography ($CHCl_3$) to give **30** as an off-white oil (302 mg, 92%): IR (KBr) 2930-2860 (aliph CH), 1725 (C=O), 1676 (C=O), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.19 (6H, s, $Si(CH_3)_2$), 0.98 (9H, s, $C(CH_3)_3$), 1.19 (3H, s, C-18-H_3), 1.29-3.02 (11H, m), 2.80-2.86 (2H, m, C-6-H_2), 4.33 (1H, app dd, J = 14.8 Hz, J = 5.8 Hz, N-CH_AH_B), 4.42 (1H, app dd, J = 14.8 Hz, J = 5.8 Hz, N-CH_AH_B), 5.11-5.19 (2H, m, C-3'-H_2), 5.74-5.85 (1H, m, C-2'-H), 6.56 (1H, d, J = 2.7 Hz, C-4-H), 6.64 (1H, dd, J = 8.3 Hz, J = 2.7 Hz, C-2-H) and 7.13 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 454.3 [100, $(M+H)^+$], 396.2 [35, $(M+H-C(CH_3)_3)^+$], 72.9 [54]; MS *m/z* (FAB-) 606.3 [32, $(M+NBA)^-$], 452.2 [100, $(M-H)^-$], 412.2 [56, $(M-H-C_3H_4)^-$]; Acc MS *m/z* (FAB+) 454.2760, $C_{27}H_{40}NO_5Si$ requires 454.2777.

3-Hydroxy-*N*-allyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (31)

Tetrabutyl ammonium fluoride trihydrate (183 mg, 701 μmol) was added to a stirred solution of **30** (265 mg, 584 μmol) in THF (10 mL) at room temperature under an atmosphere of N_2. After stirring for 2 hours, another 1.2 eq. of TBAF trihydrate was

added and the reaction was quenched 5 hours later with H_2O (40 mL) and acidified with 5M HCl. The resulting white precipitate was filtered, washed and air dried to give **31** as a white powder (172 mg, 87%). This product was recrystallised from EtOAc to give white crystals (114 mg, 58%): mp 246-248°C [lit.[160] (MeOH) 238-240°C]; IR (KBr) 3445 (OH), 2920-2860 (aliph CH), 1720 (C=O), 1660 (C=O), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.18 (3H, s, C-18-H_3), 1.30-3.02 (11H, m), 2.82-2.87 (2H, m, C-6-H_2), 4.33 (1H, app ddt, J_{BA} = 14.8 Hz, J = 5.6 Hz, J = 1.4 Hz, N-C$H_A$$H_B$), 4.41 (1H, app ddt, J_{AB} = 14.8 Hz, J = 5.6 Hz, J = 1.4 Hz, N-C$H_A$$H_B$), 4.72 (1H, s, exchanged with D_2O, OH), 5.12-5.19 (2H, m, C-3'-H_2), 5.75-5.85 (1H, m, C-2'-H), 6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.65 (1H, dd, J = 8.3 Hz, $J_{C\text{-}4\text{-}H,C\text{-}2\text{-}H}$ = 2.7 Hz, C-2-H) and 7.17 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 340.2 [100, $(M+H)^+$]; MS *m/z* (FAB-) 491.1 [50, $(M-H+NBA)^-$], 338.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 340.1916, $C_{21}H_{26}NO_5$ requires 340.1913. HPLC ($MeOH/H_2O$, 70:30; λ_{max} = 279.3 nm) Rt = 3.91 min, 100%. Found: C, 73.90; H, 7.37; N, 4.11. $C_{21}H_{25}NO_5$ requires: C, 74.31; H, 7.42; N, 4.13%.

3-Sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (32)

Following method 3, reaction of **17** (100 mg, 334 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 4 hours. The crude product was washed with boiling acetone and the precipitate was filtered to give **32** as a white solid (56 mg, 44%): mp 242-244°C; IR (KBr) 3250 (NH_2), 3090 (arom CH), 2940-2850 (aliph CH), 1690 (C=O), 1695 (C=O), 1640-1560 (arom C=C), 1370 (SO_2), 1170 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.10 (3H, s, C-18-H_3), 1.19-2.72 (11H, m), 2.81-2.85 (2H, m, C-6-H_2), 6.98 (1H, d, J = 2.3 Hz, C-4-H), 7.03 (1H, dd, J = 8.6 Hz, J = 2.3 Hz, C-2-H), 7.38 (1H, d, J = 8.6 Hz, C-1-H), 7.91 (2H, s, exchanged with D_2O, NH_2) and 10.65 (1H, s, exchanged with D_2O, NH); δ_C (DMSO-d_6, 100.4 MHz) 16.2 (q, C-18), 25.0 (2×t), 29.0 (t), 32.4 (t), 32.7 (t), 37.5 (d), 40.3 (d), 40.5 (s, C-13), 42.1 (d), 119.2 (d), 121.5 (d), 126.4 (d), 137.5 (s), 137.6 (s), 147.8 (s, C-3), 172.1 (s, C=O) and 178.9 (s, C=O); MS *m/z* (FAB+) 379.3 [94, $(M+H)^+$], 157.2 [32], 133.2 [56], 97.2 [100], 82.2 [28]; MS *m/z* (FAB-) 531.2 [37, $(M+NBA)^-$], 377.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 379.1331, $C_{18}H_{23}N_2O_5S$ requires 379.1328. HPLC ($MeOH/H_2O$, 50:50; λ_{max} = 266.3 nm) Rt = 5.70 min, 100%. Found: C, 56.80; H, 5.83; N, 7.19. $C_{18}H_{22}N_2O_5S$ requires: C, 57.13; H, 5.86; N, 7.40%.

3-Sulfamoyloxy-*N*-methyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (33)

Following method 3, reaction of **20** (100 mg, 319 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 3 hours. The crude product was recrystallised from $CHCl_3$ to give **33** as white crystals (84 mg, 67%): mp 219-222°C; IR (KBr) 3300, 3230 (NH_2), 3100 (arom CH), 2945-2865 (aliph CH), 1710 (C=O), 1655 (C=O), 1605-1500 (arom C=C), 1390 (SO_2), 1190 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.18 (3H, s, C-18-H_3), 1.32-3.02 (11H, m), 2.89-2.93 (2H, m, C-6-H_2), 3.16 (3H, s, N-CH_3), 4.85 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, J = 2.3 Hz, C-4-H), 7.11 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H) and 7.33 (1H, d, J = 8.5 Hz, C-1-H); MS *m/z* (FAB+) 393.0 [100, $(M+H)^+$], 109.0 [43], 81.0 [64, $(SO_2NH_2+H)^+$]; MS *m/z* (FAB-) 545.3 [38, $(M+NBA)^-$], 391.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 393.1472, $C_{19}H_{25}N_2O_5S$ requires 393.1484. HPLC (MeOH/H_2O, 60:40; λ_{max} = 266.3 nm) Rt = 4.72 min, 100%. Found: C, 58.30; H, 6.17; N, 7.19. $C_{19}H_{24}N_2O_5S$ requires: C, 58.15; H, 6.16; N, 7.14%.

3-Sulfamoyloxy-*N*-ethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (34)

Following method 3, reaction of **19** (70 mg, 214 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 1.5 hours. The crude product was recrystallised from EtOAc/hexane (1:2) to give **34** as off-white crystals (72 mg, 83%): mp 215-217°C; IR (KBr) 3415, 3305 (NH_2), 2970-2870 (aliph CH), 1715 (C=O), 1665 (C=O), 1375 (SO_2), 1190 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.11 (3H, t, J = 7.0 Hz, C-2'-H_3), 1.17 (3H, s, C-18-H_3), 1.24-2.99 (11H, m), 2.88-2.95 (2H, m, C-6-H_2), 3.74-3.88 (2H, m, N-CH_2), 4.89 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, J = 2.4 Hz, C-4-H), 7.12 (1H, dd, J = 8.5 Hz, J = 2.4 Hz, C-2-H) and 7.33 (1H, d, J = 8.5 Hz, C-1-H); MS *m/z* (FAB+) 813.2 [40, $(2M+H)^+$], 560.1 [70, $(M+H+NBA)^+$], 407.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 811.4 [72, $(2M-H)^-$], 712.3 [47, $(M+2NBA)^-$], 559.2 [30, $(M+NBA)^-$], 405.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 407.1645, $C_{20}H_{27}N_2O_5S$ requires 407.1641.

3-Sulfamoyloxy-*N*-propyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (35)

Following method 3, reaction of **20** (100 mg, 293 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 6 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 95:5) to give **35** as a white solid (107 mg, 87%).

An analytical sample was recrystallised from acetone/hexane (1:2) to give white crystals: mp 202-204°C; IR (KBr) 3365, 3255 (NH_2), 3095 (arom CH), 2965-2880 (aliph CH), 1710 (C=O), 1660 (C=O), 1600-1500 (arom C=C), 1380 (SO_2), 1180 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.90 (3H, t, *J* = 7.4 Hz, C-3′-H_3), 1.17 (3H, s, C-18-H_3), 1.32-3.00 (13H, m), 2.88-2.93 (2H, m, C-6-H_2), 3.64-3.80 (2H, m, N-CH_2), 4.90 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, *J* = 2.5 Hz, C-4-H), 7.11 (1H, dd, *J* = 8.4 Hz, *J* = 2.5 Hz, C-2-H) and 7.33 (1H, d, *J* = 8.4 Hz, C-1-H); MS *m/z* (FAB+) 421.0 [100, $(M+H)^+$], 109.0 [52], 97.0 [45], 81.0 [74, $(SO_2NH_2+H)^+$], 67.0 [60]; MS *m/z* (FAB-) 573.3 [34, $(M+NBA)^-$], 419.3 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 421.1800, $C_{21}H_{29}N_2O_5S$ requires 421.1797. HPLC ($MeOH/H_2O$, 70:30; λ_{max} = 266.3 nm) Rt = 4.61 min, 100%. Found: C, 60.00; H, 6.60; N, 6.49. $C_{21}H_{28}N_2O_5S$ requires: C, 59.98; H, 6.71; N, 6.66%.

3-Sulfamoyloxy-*N*-butyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (36)

Following method 3, reaction of **21** (90 mg, 253 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 1.5 hours. The crude product was recrystallised from acetone/hexane (1:2) to give **36** as white crystals (78 mg, 71%): mp 194-196°C; IR (KBr) 3335, 3250 (NH_2), 2940-2870 (aliph CH), 1710 (C=O), 1650 (C=O), 1385 (SO_2), 1190 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, t, *J* = 7.2 Hz, C-4′-H_3), 1.17 (3H, s, C-18-H_3), 1.25-2.99 (15H, m), 2.88-2.92 (2H, m, C-6-H_2), 3.75 (2H, m, N-CH_2), 4.91 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, *J* = 2.3 Hz, C-4-H), 7.12 (1H, dd, *J* = 8.7 Hz, *J* = 2.3 Hz, C-2-H) and 7.34 (1H, d, *J* = 8.7 Hz, C-1-H); MS *m/z* (FAB+) 869.2 [78, $(2M+H)^+$], 588.1 [78, $(M+H+NBA)^+$], 435.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 587.2 [32, $(M+NBA)^-$], 433.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 435.1960, $C_{22}H_{31}N_2O_5S$ requires 435.1954. Found: C, 60.50; H, 6.86; N, 6.26. $C_{22}H_{30}N_2O_5S$ requires: C, 60.81; H, 6.96; N, 6.45%.

3-Sulfamoyloxy-*N*-pentyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (37)

Following method 3, reaction of **22** (100 mg, 271 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 3.5 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 95:5) to give **37** as a white foam (111 mg, 92%). An analytical sample was recrystallised from EtOAc/hexane (1:2) to give white crystals: mp 159-161°C; IR (KBr) 3345, 3255 (NH_2), 3095 (arom CH), 2930-

2870 (aliph CH), 1720 (C=O), 1655 (C=O), 1600-1500 (arom C=C), 1385 (SO_2), 1190 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 100 MHz) 0.89 (3H, t, J = 7.4 Hz, C-5′-H_3), 1.17 (3H, s, C-18-H_3), 1.21-2.98 (17H, m), 2.90-2.94 (2H, m, C-6-H_2), 3.66-3.81 (2H, m, N-CH_2), 4.94 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, J = 2.5 Hz, C-4-H), 7.11 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H) and 7.33 (1H, d, J = 8.6 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 13.9 (q, C-5′), 16.4 (q, C-18), 22.3 (t), 25.3 (2×t), 27.5 (t), 29.0 (t), 29.3 (t), 33.5 (2×t), 38.1 (d), 40.0 (t, C-1′), 40.2 (d), 41.2 (s, C-13), 42.5 (d), 119.0 (d), 121.6 (d), 126.5 (d), 138.0 (s), 138.1 (s), 147.8 (s, C-3), 171.3 (s, C=O) and 178.1 (s, C=O); MS *m/z* (FAB+) 449.0 [100, $(M+H)^+$], 133.0 [33], 111.0 [32], 97.0 [46]; MS *m/z* (FAB-) 601.4 [34, $(M+NBA)^-$], 447.3 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 449.2111, $C_{23}H_{33}N_2O_5S$ requires 449.2110. HPLC (MeOH/H_2O, 80:20; λ_{max} = 266.3 nm) Rt = 3.70 min, 98%. Found: C, 61.70; H, 7.30; N, 6.22. $C_{23}H_{32}N_2O_5S$ requires: C, 61.58; H, 7.19; N, 6.24%.

3-Sulfamoyloxy-*N*-hexyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (38)

Following method 3, reaction of **23** (130 mg, 339 μmol) with sulfamoyl chloride in DMA (2.5 mL) was complete within 2 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 9:1) to give **38** as a white foam (127 mg, 81%). This compound was recrystallised from EtOAc/hexane (1:2) to give colourless crystals (77 mg, 49%): mp 112-115°C; IR (KBr) 3310, 3190 (NH_2), 2925-2860 (aliph CH), 1720 (C=O), 1655 (C=O), 1390 (SO_2), 1185 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.88 (3H, t, J = 6.6 Hz, C-6′-H_3), 1.19 (3H, s, C-18-H_3), 1.24-2.99 (19H, m), 2.88-2.94 (2H, m, C-6-H_2), 3.66-3.82 (2H, m, N-CH_2), 4.91 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, J = 2.5 Hz, C-4-H), 7.11 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H) and 7.33 (1H, d, J = 8.6 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 14.1 (q, C-6′), 16.6 (q, C-18), 22.6 (t), 25.5 (t), 26.7 (t), 27.9 (t), 29.5 (t), 31.5 (2×t), 33.6 (t), 33.7 (t), 38.2 (d), 40.2 (t, C-1′), 40.3 (d), 41.4 (s, C-13), 42.6 (d), 119.2 (d), 121.7 (d), 126.6 (d), 138.1 (s), 138.3 (s), 147.9 (s, C-3), 171.5 (s, C=O) and 178.2 (s, C=O); MS *m/z* (FAB+) 925.3 [64, $(2M+H)^+$], 463.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 1077.5 [70, $(2M+NBA)^-$], 615.3 [70, $(M+NBA)^-$], 462.2 [100, M^-]; Acc MS *m/z* (FAB+) 463.2263, $C_{24}H_{35}N_2O_5S$ requires 463.2267. Found: C, 62.60; H, 7.43; N, 6.20. $C_{24}H_{34}N_2O_5S$ requires: C, 62.31; H, 7.41; N, 6.06%.

3-Sulfamoyloxy-*N*-(4′-bromobutyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (39)

Following method 4, a solution of **24** (130 mg, 299 μmol) in anhydrous DMF (2 mL) was treated with NaH (14 mg, 359 μmol) and the subsequent reaction with sulfamoyl chloride (6 eq.) was complete within 2 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 9:1) to give **39** as a white foam (154 mg, 100%). This compound was recrystallised from EtOAc/hexane (1:2) to give white crystals (125 mg, 81%): mp 162-165°C; IR (KBr) 3380, 3260 (NH_2), 2945-2870 (aliph CH), 1720 (C=O), 1650 (C=O), 1565-1495 (arom C=C), 1388 (SO_2), 1180 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.18 (3H, s, C-18-H_3), 1.22-3.00 (15H, m), 2.86-2.97 (2H, m, C-6-H_2), 3.42 (2H, t, *J* = 6.6 Hz, C-4′-H_2), 3.79 (2H, m, N-CH_2), 4.89 (2H, s, exchanged with D_2O, NH_2), 7.06 (1H, d, *J* = 2.5 Hz, C-4-H), 7.12 (1H, dd, *J* = 8.6 Hz, *J* = 2.5 Hz, C-2-H) and 7.33 (1H, d, *J* = 8.6 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 16.6 (q, C-18), 25.4 (t), 25.5 (t), 26.7 (t), 29.4 (t), 30.1 (t), 33.2 (t), 33.5 (t), 33.7 (t), 38.2 (d), 39.1 (t, C-1′), 40.3 (d), 41.4 (s, C-13), 42.6 (d), 119.2 (d), 121.7 (d), 126.6 (d), 138.1 (s), 138.2 (s), 147.9 (s, C-3), 171.4 (s, C=O) and 178.2 (s, C=O); MS *m/z* (FAB+) 513.1 [100, $(M+H)^+$], 435.2 [46, $(M-Br+H)^+$]; Acc MS *m/z* (FAB+) 515.1038, $C_{22}H_{30}{}^{81}BrN_2O_5S$ requires 515.1038 and 513.1038, $C_{22}H_{30}BrN_2O_5S$ requires 513.1059.

3-Sulfamoyloxy-*N*-(4′-bromobutyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (39) and 3-sulfamoyloxy-*N*-(4′-chloro-butyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (40)

Following method 3, reaction of **24** (60 mg, 138 μmol) with sulfamoyl chloride in DMA (0.8 mL) was complete within 3 hours. The crude mixture was purified by flash chromatography ($CHCl_3$/acetone, 9:1) to give a white foam (66 mg) containing **39** and **40**: HPLC (MeOH/H_2O, 68:32; λ_{max} = 267.5 nm) Rt (**40**) = 5.52 min, 58% and Rt (**39**) = 6.50 min, 42%. The final product **40** was isolated after preparative HPLC (MeOH/H_2O, 68:32) to give a white solid (18 mg, 28%): mp 162-164°C; IR (KBr) 3390, 3245 (NH_2), 2940-2865 (aliph CH), 1720 (C=O), 1645 (C=O), 1560-1490 (arom C=C), 1385 (SO_2), 1185 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.17 (3H, s, C-18-H_3), 1.28-3.00 (15H, m), 2.88-2.96 (2H, m, C-6-H_2), 3.54 (2H, t, *J* = 6.4 Hz, C-

4′-H_2), 3.79 (2H, m, N-CH_2), ~4-5 (2H, br s, NH_2), 7.06 (1H, m, C-4-H), 7.11 (1H, app dd, *J* = 8.5 Hz, *J* = 2.1 Hz, C-2-H) and 7.33 (1H, d, *J* = 8.5 Hz, C-1-H); MS *m/z* (FAB+) 469.3 [100, $(M+H)^+$]; MS *m/z* (FAB-) 467.2 [100, $(M-H)^-$], 621.3 [40, $(M+NBA)^-$]; Acc MS *m/z* (FAB+) 469.1540, $C_{22}H_{30}ClN_2O_5S$ requires 469.1564.

3-Sulfamoyloxy-*N*-cyclopropylmethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (41)

Following method 3, reaction of **25** (100 mg, 283 μmol) with sulfamoyl chloride in DMA (1 mL) was complete within 1.5 hours. The crude product was recrystallised from acetone/hexane (1:2) to give **41** as white crystals (112 mg, 92%): mp 202-204°C; IR (KBr) 3280 (NH_2), 2960 (aliph CH), 1700 (C=O), 1660 (C=O), 1395 (SO_2), 1185 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.29-0.34 (2H, m, C-3′-H_2), 0.40-0.45 (2H, m, C-4′-H_2), 1.08-1.16 (1H, m, C-1′-H), 1.19 (3H, s, C-18-H_3), 1.32-3.02 (11H, m), 2.88-2.96 (2H, m, C-6-H_2), 3.66 (2H, m, N-CH_2), 4.93 (2H, s, exchanged with D_2O, NH_2), 7.07 (1H, d, *J* = 2.5 Hz, C-4-H), 7.12 (1H, dd, *J* = 8.6 Hz, *J* = 2.5 Hz, C-2-H) and 7.34 (1H, d, *J* = 8.6 Hz, C-1-H); MS *m/z* (FAB+) 865.1 [55, $(2M+H)^+$], 586.1 [45, $(M+H+NBA)^+$], 433.0 [100, $(M+H)^+$]; MS *m/z* (FAB-) 585.2 [30, $(M+NBA)^-$], 431.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 433.1794, $C_{22}H_{29}N_2O_5S$ requires 433.1797. Found: C, 61.00; H, 6.85; N, 5.91. $C_{22}H_{28}N_2O_5S$ requires: C, 61.09; H, 6.52; N, 6.48%.

3-Sulfamoyloxy-*N*-(4″-*tert*-butyl-benzyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (42)

Following method 3, reaction of **26** (200 mg, 449 μmol) with sulfamoyl chloride in DMA (2 mL) was complete within 6.5 hours. The crude product was recrystallised from EtOAc/hexane (1:2) to give **42** as white crystals (199 mg, 85%): mp 227-230°C; IR (KBr) 3320, 3240 (NH_2), 2960-2870 (aliph CH), 1720 (C=O), 1660 (C=O), 1385 (SO_2), 1180 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.16 (3H, s, C-18-H_3), 1.29 (9H, s, $C(CH_3)_3$), 1.30-3.02 (11H, m), 2.87-2.93 (2H, m, C-6-H_2), 4.87 (2H, s, exchanged with D_2O, NH_2), 4.87-4.96 (2H, m, N-CH_AH_B) 7.06 (1H, d, *J* = 2.5 Hz, C-4-H), 7.11 (1H, dd, *J* = 8.6 Hz, *J* = 2.5 Hz, C-2-H) and 7.24-7.34 (5H, m, C-1-H, C-2″-H, C-3″-H, C-5″-H and C-6″-H); δ_C ($CDCl_3$, 100.4 MHz) 16.4 (q, C-18), 25.3

(2×t), 29.3 (t), 31.2 (3×q, C(CH_3)$_3$), 33.5 (2×t), 34.4 (s, C(CH$_3$)$_3$), 38.0 (d), 40.0 (d), 41.3 (s, C-13), 42.5 (d), 42.8 (t, C-1′), 119.0 (d), 121.6 (d), 125.1 (2×d), 126.5 (d), 127.8 (2×d), 133.9 (s), 137.9 (s), 138.1 (s), 147.8 (s), 149.8 (s, C-3), 171.2 (s, C=O) and 178.1 (s, C=O); MS *m/z* (FAB+) 1049.3 [70, $(2M+H)^+$], 525.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 1047.5 [80, $(2M-H)^-$], 523.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 525.2405, $C_{29}H_{37}N_2O_5S$ requires 525.2423.

3-Sulfamoyloxy-*N*-(1″-pyridin-3″-ylmethyl)-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (43)

Following method 4, a solution of **27** (45 mg, 115 μmol) in anhydrous DMF (0.5 mL) was treated with NaH (5 mg, 230 μmol) and the subsequent reaction with sulfamoyl chloride (6 eq.) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 8:2) to give **42** as an off-white powder (42 mg, 78%). This compound was recrystallised from acetone/hexane (1:2) to give colourless crystals (28 mg, 52%): mp 215-218°C. IR (KBr) 3335 (NH_2), 3100-2850 (aliph CH), 1720 (C=O), 1675 (C=O), 1380 (SO_2), 1190 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.10 (3H, s, C-18-H_3), 1.15-2.97 (11H, m), 2.79-2.84 (2H, m, C-6-H_2), 4.81 (1H, d, J_{BA} = 14.8 Hz, N-CH_AH$_B$), 4.86 (1H, d, J_{AB} = 14.8 Hz, N-CH$_A$$H_B$), 6.96 (1H, d, J = 2.7 Hz, C-4-H), 7.01 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.31 (1H, dd, J = 7.8 Hz, J = 4.7 Hz, C-5″-H), 7.36 (1H, d, J = 8.6 Hz, C-1-H), 7.57 (1H, m, C-4″-H), 7.89 (2H, s, exchanged with D_2O, NH_2) and 8.41-8.44 (2H, m, C-2″-H and C-6″-H); MS *m/z* (FAB+) 470.3 [48, $(M+H)^+$], 133.2 [38], 111.2 [52], 97.1 [100]; MS *m/z* (FAB-) 622.3 [52, $(M+NBA)^-$], 468.3 [100, $(M-H)^-$], 276.2 [62], 198 [48], 139.1 [46], 93.1 [40]; Acc MS *m/z* (FAB+) 470.1767, $C_{24}H_{28}N_3O_5S$ requires 470.1750. HPLC (MeOH/H_2O, 60:40; λ_{max} = 260.4 nm) Rt = 4.84 min, 100%. Found: C, 60.00; H, 5.86; N, 8.57. $C_{24}H_{27}N_3O_5S.(H_2O)_{1/2}$ requires: C, 60.03; H, 5.90; N, 8.78%.

3-Sulfamoyloxy-*N*-benzyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (44)

Following method 3, reaction of **28** (150 mg, 385 μmol) with sulfamoyl chloride in DMA (1.5 mL) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 9:1) to give **44** as a white powder (151 mg,

84%). This compound was recrystallised from acetone/hexane (1:2) to give white crystals (133 mg, 74%): mp 208-210°C; IR (KBr) 3340, 3230 (NH_2), 3100-3050 (arom CH), 2950-2870 (aliph CH), 1715 (C=O), 1655 (C=O), 1610-1495 (arom C=C), 1385 (SO_2), 1195 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.13 (3H, s, C-18-H_3), 1.17-2.96 (11H, m), 2.81-2.87 (2H, m, C-6-H_2), 4.80 (1H, d, J_{BA} = 14.6 Hz, N-CH_AH$_B$), 4.86 (1H, d, J_{AB} = 14.6 Hz, N-CH$_A$$H_B$), 6.99 (1H, d, J = 2.3 Hz, C-4-H), 7.04 (1H, dd, J = 8.4 Hz, J = 2.3 Hz, C-2-H), 7.19-7.40 (6H, m, C_6H_5 and C-1-H) and 7.92 (2H, s, exchanged with D_2O, NH_2); MS *m/z* (FAB+) 469.2 [100, $(M+H)^+$]; MS *m/z* (FAB-) 621.3 [38, $(M+NBA)^-$], 467.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 469.1789, $C_{25}H_{29}N_2O_5S$ requires 469.1797. HPLC (MeOH/H_2O, 70:30; λ_{max} = 266.3 nm) Rt = 5.14 min, 100%. Found: C, 63.90; H, 6.12; N, 5.86. $C_{25}H_{28}N_2O_5S$ requires: C, 64.08; H, 6.02; N, 5.98%.

3-Sulfamoyloxy-*N*-allyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (45)

Following method 3, reaction of **31** (150 mg, 345 μmol) with sulfamoyl chloride in DMA (2 mL) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 9:1) to give **45** as a white foam (85 mg, 99%). This compound was recrystallised from acetone/hexane (1:2) to give white crystals (75 mg, 87%): mp 210-213°C; IR (KBr) 3385, 3275 (NH_2), 2935-2870 (aliph CH), 1715 (C=O), 1670 (C=O), 1600-1495 (arom C=C), 1385 (SO_2), 1185 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.13 (3H, s, C-18-H_3), 1.25-2.89 (11H, m), 2.81-2.87 (2H, m, C-6-H_2), 4.17 (1H, app ddt, J = 15.0 Hz, J = 5.1 Hz, J = 1.7 Hz, N-CH_AH$_B$), 4.27 (1H, app ddt, J = 15.0 Hz, J = 5.1 Hz, J = 1.7 Hz, N-CH$_A$$H_B$), 4.97-5.08 (2H, m, C-3′-H_2), 5.69-5.79 (1H, m, C-2′-H), 6.99 (1H, d, J = 2.5 Hz, C-4-H), 7.04 (1H, dd, J = 8.6 Hz, J = 2.5 Hz, C-2-H), 7.38 (1H, d, J = 8.6 Hz, C-1-H) and 7.91 (2H, s, exchanged with D_2O, NH_2); δ_C (DMSO-d_6, 100.4 MHz) 16.3 (q, C-18), 24.8 (t), 25.0 (t), 29.0 (t), 32.8 (t), 33.5 (t), 37.4 (d), ~39 (d, under solvent peaks), 40.9 (s, C-13), 41.1 (t, C-1′), 42.0 (d), 115.6 (t, C-3′), 119.2 (d), 121.5 (d), 126.4 (d), 132.7 (d), 137.5 (s), 137.6 (s), 147.8 (s, C-3), 170.8 (s, C=O) and 177.6 (s, C=O); MS *m/z* (FAB+) 837.4 [48, $(2M+H)^+$], 572.2 [68, $(M+H+NBA)^+$], 419.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 571.1 [30, $(M+NBA)^-$], 417.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 419.1635, $C_{21}H_{27}N_2O_5S$ requires 419.1641. HPLC (MeOH/H_2O, 70:30; λ_{max} = 266.3

nm) Rt = 3.25 min, 100%. Found: C, 60.30; H, 6.32; N, 6.56. $C_{21}H_{26}N_2O_5S$ requires: C, 60.27; H, 6.26; N, 6.69%.

5.4.2 Further D-ring modifications

3-Benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-amine (46)

A solution of **5** (100 mg, 257 μmol) in anhydrous THF (3.5 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (78 mg, 2.06 mmol) in anhydrous THF (3.5 mL) at 0°C under an atmosphere of N_2. The resulting mixture was allowed to warm to room temperature and heated to reflux for 48 hours. After cooling down, H_2O (300 μL) was added, followed by 10% aq. NaOH (300 μL) and another portion of H_2O (900 μL). The mixture was filtered, the filter cake was washed with THF and the filtrate was concentrated under reduced pressure to give a brown product. The crude material was purified by flash chromatography ($CHCl_3$/MeOH, 98:2 to 75:25, gradient) to give **46** as an off-white solid (7 mg, 7%): mp 222-225°C; IR (KBr) 3410 (NH), 2950-2810 (aliph CH), 1615-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.18 (3H, s, C-18-H_3), 1.22-2.87 (14H, m), 2.63 (1H, d, J_{AB} = 12.3 Hz, N-CH_AH_B), 3.06 (1H, d, J_{BA} = 12.3 Hz, N-CH_AH_B), 3.57-3.63 (1H, m, N-$CH_A'H_B'$), 5.03 (2H, s, OCH_2Ar), 6.71 (1H, d, J = 2.6 Hz, C-4-H), 6.79 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.18 (1H, d, J = 8.6 Hz, C-1-H) and 7.29-7.43 (5H, m, C_6H_5), NH not seen; MS *m/z* (FAB+) 362.2 [100, $(M+H)^+$], 91.1 [38, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 362.2498, $C_{25}H_{32}NO$ requires 362.2484.

3-Benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-dithioimide (47) and 3-benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16-one,17-thioimide (48)

Lawesson's reagent (4.5 g, 11.17 mmol) was added to a stirred suspension of **5** (2.9 g, 7.45 mmol) in anhydrous toluene (120 mL), under an atmosphere of N_2. The resulting mixture was heated to reflux for 4 hours giving a yellow solution. After cooling down, the solvent was removed under reduced pressure and the yellow crude mixture was purified by flash chromatography ($CHCl_3$/Hexane, 7:3 to $CHCl_3$, gradient) to give two products.

The less polar fraction gave **47** as a yellow solid (~1.5 g) which was recrystallised from EtOH to give yellow crystals (1.13 g, 36%): mp 209-211°C; IR (KBr) 3120 (NH), 2940-2860 (aliph CH), 1610-1600 (arom C=C), 1500 (C=S) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.25 (3H, s, C-18-H_3), 1.30-2.55 (9H, m), 2.65 (1H, dd, *J* = 19.5 Hz, *J* = 12.9 Hz, C-15-H), 2.84-2.90 (2H, m, C-6-H_2), 3.56 (1H, dd, *J* = 19.5 Hz, *J* = 4.3 Hz, C-15-H), 5.04 (2H, s, OCH_2Ar), 6.72 (1H, d, *J* = 2.7 Hz, C-4-H), 6.81 (1H, dd, *J* = 8:6 Hz, *J* = 2.7 Hz, C-2-H), 7.22 (1H, d, *J* = 8.6 Hz, C-1-H), 7.30-7.44 (5H, m, C_6H_5) and 10.63 (1H, s, exchanged with D_2O, NH); δ_C ($CDCl_3$, 100.4 MHz) 19.7 (q, C-18), 26.0 (t), 26.2 (t), 29.8 (t), 37.6 (t), 39.3 (d), 41.6 (t), 42.2 (2×t), 46.1 (s, C-13), 69.9 (t, OCH_2Ar), 112.5 (d), 114.4 (d), 126.2 (d), 127.3 (2×d), 127.8 (d), 128.4 (2×d), 131.4 (s), 136.9 (s), 137.1 (s), 156.8 (s, C-3), 202.0 (s, C=S) and 214.0 (s, C=S); MS *m/z* (FAB+) 422.1 [40, $(M+H)^+$], 90.9 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 422.1620, $C_{25}H_{28}NOS_2$ requires 422.1612.

The more polar fraction gave **48** as a pale yellow solid (~2 g) which was recrystallised from EtOH to give yellow crystals (1.74 g, 58%): mp 204-207°C; IR (KBr) 3230 (NH), 2940-2860 (aliph CH), 1720 (C=O), 1605-1470 (C=S and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.21 (3H, s, C-18-H_3), 1.30-2.66 (9H, m), 2.62 (1H, dd, *J* = 19.4 Hz, *J* = 12.7 Hz, C-15-H), 2.83-2.89 (2H, m, C-6-H_2), 3.49 (1H, dd, *J* = 19.4 Hz, *J* = 3.9 Hz, C-15-Hβ), 5.02 (2H, s, OCH_2Ar), 6.71 (1H, d, *J* = 2.7 Hz, C-4-H), 6.80 (1H, dd, *J* = 8.6 Hz, *J* = 2.7 Hz, C-2-H), 7.20 (1H, d, *J* = 8.6 Hz, C-1-H), 7.28-7.43 (5H, m, C_6H_5) and 9.55 (1H, s, exchanged with D_2O, NH); δ_C ($CDCl_3$, 100.4 MHz) 16.6 (q, C-18), 25.7 (t), 26.3 (t), 30.0 (t), 33.1 (t), 38.5 (d), 40.9 (s, C-13), 41.8 (d), 42.8 (d), 43.0 (d), 70.2 (t, OCH_2Ar), 112.9 (d), 114.8 (d), 126.5 (d), 127.6 (2×d), 128.1 (d), 128.8 (2×d), 131.7 (s), 137.3 (s), 137.5 (s), 157.1 (s, C-3), 175.7 (s, C=O) and 207.6 (s, C=S); MS *m/z* (FAB+) 406.0 [59, $(M+H)^+$], 90.9 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 406.1846, $C_{25}H_{28}NO_2S$ requires 406.1841.

3-Hydroxy-*N*-ethyl-16,17-*seco*-estra-1,3,5(10)-triene-16,17-amine (49)

A suspension of Raney nickel (50% slurry in H_2O, 5 g) was added to a stirred solution of **47** (200 mg, 474 μmol) in EtOH/THF (1:1, 60 mL) at room temperature. The resulting suspension was stirred at room temperature overnight, after which it

was filtered through celite. The filter cake was washed with several portions of EtOH and THF, and the filtrate was concentrated under reduced pressure to give a light brown solid. The crude product was recrystallised from acetone/hexane to give **49** as off-white crystals (34 mg, 24%): mp 188-191°C; IR (KBr) 3100 (OH), 2925-2825 (aliph CH), 1610-1505 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.95 (3H, s, C-18-H_3), 0.99 (3H, t, J = 7.2 Hz, C-2'-H_2), 1.10-2.73 (18H, m), 2.95-3.01 (1H, m, N-CH_AH_B), 6.44 (1H, d, J = 2.7 Hz, C-4-H), 6.52 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.06 (1H, d, J = 8.6 Hz, C-1-H) and 9.02 (1H, s, exchanged with D_2O, OH); MS *m/z* (FAB+) 300.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 298.3 [100, $(M-H)^-$].

3-Hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-17-one-16,17-amide (50)

A suspension of Raney nickel (50% slurry in H_2O, 5 g) was added to a stirred solution of **48** (300 mg, 740 µmol) in EtOH/THF (1:1, 60 mL) at room temperature. The resulting suspension was stirred at room temperature for 7 hours, after which it was filtered through celite. The filter cake was washed with several portions of EtOH and THF, and the filtrate was concentrated under reduced pressure to give a white solid. The crude product was recrystallised from EtOH to give **50** as white crystals (155 mg, 73%): mp 334-337°C (dec.); IR (KBr) 3350 (NH), 3185 (br, OH), 2945-2860 (aliph CH), 1635 (C=O), 1585-1500 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.03 (3H, s, C-18-H_3), 1.18-2.74 (13H, m), 3.08-3.23 (2H, m, N-CH_AH_B), 6.42 (1H, d, J = 2.6 Hz, C-4-H), 6.50 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.04 (1H, d, J = 8.5 Hz, C-1-H), 7.10 (1H, s, exchanged with D_2O, OH or NH) and 9.00 (1H, s, exchanged with D_2O, OH or NH); δ_C (DMSO-d_6, 100.4 MHz) 18.4 (q, C-18), 20.3 (t), 26.0 (t), 26.4 (t), 29.9 (t), 34.3 (t), 38.4 (d), 40.6 (s, C-13), 40.8 (t, C-16), 43.0 (d), 44.6 (d), 113.3 (d), 115.1 (d), 126.5 (d), 130.7 (s), 137.5 (s), 155.5 (s, C-3) and 177.4 (s, C=O); MS *m/z* (FAB+) 286.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 286.1805, $C_{18}H_{24}NO_2$ requires 286.1807.

3-Sulfamoyloxy-16,17-*seco*-estra-1,3,5(10)-triene-17-one-16,17-amide (51)

Following method 4, a solution of **50** (80 mg, 280 µmol) in anhydrous DMF (1 mL) was treated with NaH (13 mg, 0.34 µmol) and the subsequent reaction with sulfamoyl chloride (6 eq.) was complete after stirring overnight. The crude product was recrystallised from EtOAc/hexane (1:2) to give **51** as yellow crystals (22 mg,

21%): mp 217-218°C; IR (KBr) 3300 (br, NH_2, NH), 2940-2865 (aliph CH), 1644 (C=O), 1600-1490 (arom C=C), 1375 (SO_2), 1170 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.04 (3H, s, C-18-H_3), 1.18-2.88 (13H, m), 3.09-3.25 (2H, m, N-CH_AH_B), 6.95 (1H, d, J = 2.6 Hz, C-4-H), 7.00 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H), 7.12 (1H, s, exchanged with D_2O, NH), 7.34 (1H, d, J = 8.3 Hz, C-1-H) and 7.88 (2H, s, exchanged with D_2O, NH_2); MS *m/z* (FAB+) 365.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 365.1558, $C_{18}H_{25}N_2O_4S$ requires 365.1535.

3-Hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-dithioimide (52)

Boron tribromide (1.0 M solution in DCM, 889 µL) was added dropwise to a stirred solution of **47** (250 mg, 593 µmol) in anhydrous DCM (10 mL) at -78°C under an atmosphere of N_2. The resulting brown solution was stirred at -78°C for 4 hours, after which H_2O (50 mL) was added and the mixture was allowed to warm to room temperature. The organics were extracted with DCM (50 mL), washed with sat. $NaHCO_3$ (4×30 mL), H_2O (30 mL), then brine (30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a yellow product. The crude material was purified by flash chromatography ($CHCl_3$) to give **52** as a yellow solid (98 mg, 50%). This compound was recrystallised from $CHCl_3$/hexane to give yellow crystals (69 mg, 35%): mp 270-272°C; IR (KBr) 3410, 3230 (OH, NH), 2925-2860 (aliph CH), 1605 (arom C=C), 1500 (C=S) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.15 (3H, s, C-18-H_3), 1.19-2.80 (12H, m), ~3.35-3.40 (~1H, under solvent peaks, C-15-H), 6.44 (1H, d, J = 2.6 Hz, C-4-H), 6.52 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H), 7.06 (1H, d, J = 8.3 Hz, C-1-H), 9.03 (1H, s, exchanged with D_2O, C-3-OH) and 13.37 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 332.0 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 332.1141, $C_{18}H_{22}NOS_2$ requires 332.1143.

3-Hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-16-one,17-thioimide (53)

Boron tribromide (1.0 M solution in DCM, 1.29 mL) was added dropwise to a stirred solution of **48** (350 mg, 863 µmol) in anhydrous DCM (15 mL) at -78°C under an atmosphere of N_2. The resulting brown solution was stirred at -78°C for 5 hours, after which H_2O (50 mL) was added and the mixture allowed to warm to room temperature. The organics were extracted with DCM (50 mL), washed with sat. $NaHCO_3$ (4×30 mL), H_2O (30 mL), then brine (30 mL), dried (Na_2SO_4), filtered and

concentrated under reduced pressure to give a brown product. The crude material was purified by flash chromatography ($CHCl_3$/EtOAc, 9:1) to give **53** as a yellow solid (89 mg, 33%). This compound was recrystallised from $CHCl_3$/hexane to give pale yellow crystals (47 mg, 17%): mp 240-242°C; IR (KBr) 3420-3200 (OH, NH), 2940-2870 (aliph CH), 1720 (C=O), 1620-1475 (C=S and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.11 (3H, s, C-18-H_3), 1.17-2.74 (12H, m), 3.27 (1H, dd, J = 19.3 Hz, J = 4.1 Hz, C-15-H), 6.44 (1H, d, J = 2.7 Hz, C-4-H), 6.52 (1H, dd, J = 8.3 Hz, J = 2.7 Hz, C-2-H), 7.06 (1H, d, J = 8.3 Hz, C-1-H), 9.03 (1H, s, exchanged with D_2O, C-3-OH) and 12.22 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 316.0 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 316.1384, $C_{18}H_{22}NO_2S$ requires 316.1371.

5.4.3 A-ring modified analogues

2-Iodo-estrone (54)

Mercuric acetate (5.89 g, 18.49 mmol) was added to a stirred solution of E1 (10 g, 36.98 mmol) in a mixture of AcOH glacial (570 mL) and THF (280 mL) warmed to 55°C. After 15 minutes, iodine (8.70 g, 34.37 mmol) was added portionwise and the resulting orange solution was stirred for 2 hours at room temperature. The final light yellow mixture was concentrated under reduced pressure and a solution of KI (5% aq., 300 mL) was added. The organics were extracted with EtOAc (2×300 mL), washed with aq. $Na_2S_2O_3$ (3×200 mL), then brine (200 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The brown crude product was recrystallised from AcOH glacial to give **54** as a pale blue powder (6.42 g, 44%) and a further crop of the product (3.00 g) was obtained from the residue of the mother liquor upon recrystallisation from EtOH (overall 'crude' yield 64%). Both crops were further recrystallised from EtOH to give light grey flaky crystals (8.20 g, overall yield 56%): mp 213-215°C (dec.) [lit.[188] (MeOH) 200-205°C]; IR (KBr) 3400 (OH), 2920-2855 (aliph CH), 1725 (C=O), 1590-1495 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.91 (3H, s, C-18-H_3), 1.36-2.57 (13H, m), 2.83-2.86 (2H, m, C-6-H_2), 5.09 (1H, s, exchanged with D_2O, OH), 6.74 (1H, s, C-4-H) and 7.52 (1H, s, C-1-H); MS *m/z* (FAB+) 396.1 [100, M^+], 85.1 [39]; Acc MS *m/z* (FAB+) 396.0568, $C_{18}H_{21}O_2I$ requires 396.0586.

2-Methoxy-estrone (55)

2-Iodoestrone **54** (4 g, 10.09 mmol) and copper chloride (452 mg, 3.36 mmol) were stirred at room temperature under an atmosphere of N_2 in anhydrous pyridine (35 mL) for 30 minutes. A freshly prepared 5.1 M solution of NaOMe (19.7 mL, 100 mmol) was then added and the resulting blue solution was heated to reflux for 45 minutes. After cooling down, the final orange solution was poured into ice and acidified with 5M HCl. The organics were extracted with EtOAc (3×200 mL), washed with aq. $NaHCO_3$ (2×200 mL), then brine (2×200 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification of the crude product by flash chromatography (EtOAc/hexane, 3:17 to 5:15, gradient) gave **55** as an off-white residue (2.58 g, 78%): mp 167-170°C [lit.[182] (MeOH) 188-191°C]; IR (KBr) 3415 (br, OH), 2930-2850 (aliph CH), 1725 (C=O), 1520-1460 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.38-2.54 (13H, m), 2.80-2.84 (2H, m, C-6-H_2), 3.86 (3H, s, OCH_3), 5.45 (1H, s, exchanged with D_2O, OH), 6.66 (1H, s, C-4-H) and 6.79 (1H, s, C-1-H); MS *m/z* (FAB+) 300.2 [100, M^+]; Acc MS *m/z* (FAB+) 300.1719, $C_{19}H_{24}O_3$ requires 300.1725.

2-Methoxy-3-benzyloxy-estra-1,3,5(10)-triene-17-one (56)

^{t}BuOK (1.07 g, 9.54 mmol) was added portionwise to a stirred solution of **55** (1.91 g, 6.36 mmol) in anhydrous DMF (20 mL) at 0°C under an atmosphere of N_2. After 2 hours, benzyl bromide (1.13 mL, 9.54 mmol) was added and the mixture was stirred at room temperature for another 2 hours. The resulting orange solution was poured into H_2O (50 mL) and the organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×50 mL), brine (2×50 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was recrystallised from EtOH to give **56** as a light orange powder (2.3 g). This compound was further recrystallised from EtOH to give an off-white powder (1.81 g, 73%): mp 120-123°C [lit.[268] (MeOH) 152-154°C]; IR (KBr) 2930-2830 (aliph CH), 1735 (C=O), 1605, 1520, 1460 (arom C=C), 1255, 1205 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.36-2.55 (13H, m), 2.74-2.85 (2H, m, C-6-H_2), 3.86 (3H, s, OCH_3), 5.11 (2H, s, OCH_2Ar), 6.64 (1H, s, C-4-H), 6.84 (1H, s, C-1-H) and 7.29-7.46 (5H, m, C_6H_5); MS *m/z* (FAB+) 390.1 [88, M^+], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 390.2191, $C_{26}H_{30}O_3$ requires 390.2195.

2-Methoxy-3-benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-dioic acid (57)

This was prepared according to the procedure used for the synthesis of **4**. A solution of iodine (2.8 g, 11.1 mmol) in MeOH (35 mL) and a solution of KOH (5.0 g, 90.2 mmol) in H_2O (10 mL) and MeOH (25 mL) were added dropwise and alternatively to a stirred solution of **56** (1.52 g, 3.89 mmol) in MeOH (700 mL). After the workup, the resulting crude orange foam (1.80 g) was dissolved in a solution of KOH (2.8 g, 50.0 mmol) in MeOH/H_2O (1:2, 84 mL) and heated to reflux for 4 hours. The orange residue (4.32 g) recovered after the workup was purified by flash chromatography ($CHCl_3$/MeOH, 95:5) to give **57** as an orange powder (311 mg, 18%): mp 91-93°C; IR (KBr) 3430-3000 (br, CO_2H), 2935-2860 (aliph CH), 1705 (2×C=O), 1610-1455 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.21-2.38 (11H, m), 2.64-2.70 (2H, m, C-6-H_2), 3.72 (3H, s, OCH_3), 5.01 (2H, s, OCH_2Ar), 6.70 (1H, s, C-4-H), 6.85 (1H, s, C-1-H), 7.30-7.45 (5H, m, C_6H_5) and 12.20 (2H, br s, exchanged with D_2O, CO_2H); MS *m/z* (FAB+) 438.1 [52, M^+], 91.0 [100, $(CH_2Ar)^+$]; MS *m/z* (FAB-) 437.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 438.2055, $C_{26}H_{30}O_6$ requires 438.2042.

2-Methoxy-3-benzyloxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (58)

This was prepared according to the procedure used for the synthesis of **5** by reaction of **57** (300 mg, 684 mmol) with urea (300 mg, 4.99 mmol) at 180°C. Purification of the crude product by flash chromatography ($CHCl_3$/acetone, 95:5) gave **58** as a light yellow powder (170 mg, 59%): mp 84-87°C; IR (KBr) 3415, 3225 (br, NH), 3090-2860 (arom and aliph CH), 1720 (C=O), 1695 (C=O), 1610-1510 (arom C=C), 1260 (C-O) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.10 (3H, s, C-18-H_3), 1.14-2.66 (11H, m), 2.67-2.72 (2H, m, C-6-H_2), 3.73 (3H, s, OCH_3), 5.02 (2H, s, OCH_2Ar), 6.73 (1H, s, C-4-H), 6.86 (1H, s, C-1-H), 7.30-7.46 (5H, m, C_6H_5) and 10.64 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 419.1 [100, M^+]; Acc MS *m/z* (FAB+) 419.2110, $C_{26}H_{29}NO_4$ requires 419.2097.

2-Methoxy-3-hydroxy-16,17-*seco*-estra-1,3,5(10)-triene-16,17-imide (59)

Following method 2, a suspension of **58** (150 mg, 357 μmol) and Pd-C (10%, 80 mg) in MeOH/THF 2:1 (15 mL) was hydrogenated for 4 hours to give **59** as a light yellow powder (115 mg, 97%). An analytical sample was recrystallised from MeOH

to give white crystals: mp 202-205°C; IR (KBr) 3500-3090 (br, OH and NH), 2935-2840 (aliph CH), 1725 (C=O), 1685 (C=O), 1515-1460 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.10 (3H, s, C-18-H_3), 1.13-2.42 (11H, m), 2.63-2.69 (2H, m, C-6-H_2), 3.71 (3H, s, OCH_3), 6.45 (1H, s, C-4-H), 6.78 (1H, s, C-1-H), 8.67 (1H, s, exchanged with D_2O, OH) and 10.63 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 330.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 481.2 [43, $(M-H+NBA)^-$], 328.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 330.1695, $C_{19}H_{24}NO_4$ requires 330.1705. HPLC (MeOH/H_2O, 75:25; λ_{max} = 286.4 nm) Rt = 2.64 min, 100%.

2-Methoxy-3-sulfamoyloxy-16,17-seco-estra-1,3,5(10)-triene-16,17-imide (60)

Following method 4, a solution of **59** (70 mg, 212 µmol) in anhydrous DMF (1.5 mL) was treated with NaH (10 mg, 254 µmol) and the subsequent reaction with sulfamoyl chloride (6 eq.) was complete after stirring overnight. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 8:2) to give **60** as a white foam (68 mg, 79%). This compound was recrystallised from acetone/hexane (1:2) to give white crystals (58 mg, 67%): mp 225-230°C; IR (KBr) 3275-3160, 3065 (NH_2, NH), 2960-2820 (aliph CH), 1715 (C=O), 1695 (C=O), 1510 (arom C=C), 1375, 1180 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.11 (3H, s, C-18-H_3), 1.21-2.47 (11H, m), 2.71-2.75 (2H, m, C-6-H_2), 3.77 (3H, s, OCH_3), 7.00 (1H, s, C-4-H or C-1-H), 7.02 (1H, s, C-1-H or C-4-H), 7.84 (2H, s, exchanged with D_2O, NH_2) and 10.65 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 561.9 [24, $(M+NBA)^+$], 408.9 [100, M^+]; MS *m/z* (FAB-) 561.2 [43, $(M+NBA)^-$], 407.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 408.1345, $C_{19}H_{24}NO_6S$ requires 408.1355. HPLC (MeOH/H_2O, 90:10; λ_{max} = 279.3 nm) Rt = 1.92 min, 98%. Found: C, 56.20; H, 5.99; N, 6.83. $C_{19}H_{24}NO_6S$ requires: C, 55.87; H, 5.92; N, 6.86%.

5.4.4 16-Alkylidene derivatives of E1 and precursors

3-Acetoxy-estra-1,3,5(10)-triene-17-one (61)

AcOH glacial (60 mL, 1.05 mmol) was added dropwise over 45 minutes to a stirred solution of E1 (3 g, 11.1 mmol) in anhydrous pyridine (200 mL) at 0°C, under an atmosphere of N_2. The resulting yellow mixture was heated to reflux for 1 hour,

allowed to cool down, poured into H_2O and ice (300 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (300 mL), washed with H_2O (200 mL), aq. $NaHCO_3$ (200 mL), then brine (200 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crystalline orange crude (3.88 g) was recrystallised from IPA to give **61** as light yellow needles (3.25 g, 94%): mp 106-108°C [lit.[156] (MeOH) 120°C]; IR (KBr) 2930-2870 (aliph CH), 1765 (C=O), 1735 (C=O), 1605-1490 (arom C=C), 1210 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.91 (3H, s, C-18-H_3), 1.45-2.54 (13H, m), 2.29 (3H, s, $COCH_3$), 2.89-2.92 (2H, m, C-6-H_2), 6.81 (1H, d, J = 2.6 Hz, C-4-H), 6.85 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H) and 7.28 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 466.2 [28, $(M+H+NBA)^+$], 313.2 [100, $(M+H)^+$], 270.2 [88, $(M+H-COCH_3)^+$], 85.1 [35]; Acc MS *m/z* (FAB+) 313.1808, $C_{20}H_{25}O_3$ requires 313.1804.

3-Acetoxy-16-methylene-estra-1,3,5(10)-triene-17-one (62)

Paraformaldehyde (480 mg, 15.98 mmol of HCHO unit) and dimethylamine hydrochloride (1.6 g, 19.6 mmol) were added to a stirred solution of **61** (1.0 g, 3.20 mmol) in anhydrous isoamyl alcohol (8 mL) and the resulting mixture was heated to reflux for 24 hours. After cooling down, the final light yellow solution was poured into H_2O (30 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×50 mL), washed with $NaHCO_3$ sat. (20 mL), H_2O (20 mL), then brine (20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a light yellow oil. The residual isoamyl alcohol was distilled off using a Kugelrohr apparatus and the oily residue obtained crystallised overnight. This product was purified by flash chromatography ($CHCl_3$/EtOAc, 95:5) to give **62** as a white solid (527 mg, 51%). For analysis, a sample was triturated with hexane to give colourless crystals: mp 134-135°C; IR (KBr) 2980-2835 (aliph CH), 1765 (C=O), 1725 (C=O), 1640-1375 (arom C=C and exocyclic C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.93 (3H, s, C-18-H_3), 1.41-2.72 (11H, m), 2.29 (3H, s, $COCH_3$), 2.90-2.95 (2H, m, C-6-H_2), 5.41-5.43 (1H, m, C-1′-H), 6.09-6.12 (1H, m, C-1′-H), 6.81 (1H, d, J = 2.5 Hz, C-4-H), 6.86 (1H, dd, J = 8.3 Hz, J = 2.5 Hz, C-2-H) and 7.29 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 325.2 [100, $(M+H)^+$], 282.2 [78, $(M+H-COCH_3)^+$]; Acc MS *m/z* (FAB+) 325.1822, $C_{21}H_{25}O_3$ requires 325.1804. Found: C, 77.90; H, 7.50. $C_{21}H_{24}O_3$ requires: C, 77.75; H, 7.46%.

16-Methylene-estrone (63)

A solution of KOH (6 mg, 110 μmol) in H_2O (2 mL) was added dropwise to a stirred solution of **62** (30 mg, 92 μmol) in EtOH (10 mL). The resulting mixture was stirred at room temperature for 30 minutes, after which the solvent was removed under reduced pressure, H_2O added (20 mL) followed by 5M HCl (1 mL). The organics were extracted with EtOAc (30 mL), washed with H_2O (20 mL), then brine (20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a white product (34 mg). The crude material was recrystallised from IPA/H_2O (1:2) to give **63** as white crystals (15 mg, 58%): mp 229-231°C; IR (KBr) 3385 (OH), 2925 (aliph CH), 1715 (C=O), 1635-1450 (arom C=C and exocyclic C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.93 (3H, s, C-18-H_3), 1.39-2.72 (11H, m), 2.81-2.90 (2H, m, C-6-H_2), 4.73 (1H, s, exchanged with D_2O, OH), 5.40-5.43 (1H, m, C-1′-H), 6.09-6.11 (1H, m, C-1′-H), 6.58 (1H, d, J = 2.8 Hz, C-4-H), 6.64 (1H, dd, J = 8.3 Hz, J = 2.8 Hz, C-2-H) and 7.15 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 663.3 [65], 282.1 [72, M^+], 72.9 [100]; Acc MS *m/z* (FAB+) 282.1620, $C_{19}H_{22}O_2$ requires 282.1620.

16-Methylene-estrone (63) and 16β-ethoxymethyl-estrone (64)

A solution of KOH (41 mg, 739 μmol) in H_2O (2 mL) was added dropwise to a stirred solution of **62** (200 mg, 616 μmol) in EtOH (20 mL) at 0°C. The resulting light yellow solution was stirred for 30 minutes at 0°C, after which the solvent was removed under reduced pressure and H_2O added (40 mL). This mixture was acidified with 5M HCl and the white precipitate that formed was filtered, dried (131 mg) and purified by flash chromatography ($CHCl_3$/EtOAc, 95:5) to give two products:

The less polar fraction gave **63** as a white solid (50 mg, 29%).

The more polar fraction gave **64** as a light yellow solid (26 mg, 13%). This compound was recrystallised from EtOH/H_2O to give light yellow crystals (13 mg, 6%): mp 208-210°C; IR (KBr) 3370 (OH), 2970-2855 (aliph CH), 1730 (C=O), 1610, 1505 (arom C=C), 1225 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.89 (3H, s, C-18-H_3), 1.16 (3H, t, J = 7.0 Hz, C-2″-H_3), 1.36-2.42 (12H, m), 2.81-2.85 (2H, m, C-6-H_2), 3.42-3.50 (2H, m, C-1″-H_2), 3.61 (1H, dd, J_{AB} = 9.4 Hz, J = 3.9 Hz, C-1′-H_AH_B),

3.65 (1H, dd, J_{BA} = 9.4 Hz, J = 5.5 Hz, C-1′-H_AH_B), 4.59 (1H, s, exchanged with D_2O, OH), 6.56 (1H, d, J = 2.8 Hz, C-4-H), 6.62 (1H, dd, J = 8.5 Hz, J = 2.8 Hz, C-2-H) and 7.13 (1H, d, J = 8.5 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 12.9 (q, C-2″), 15.0 (q, C-18), 25.8 (t), 25.9 (t), 26.6 (t), 29.4 (t), 32.0 (t), 37.7 (d), 44.0 (d), 48.2 (s, C-13), 48.9 (d), 50.2 (d), 66.4 (t, C-1′ or C-1″), 69.3 (t, C-1′ or C-1″), 112.5 (d), 115.0 (d), 126.2 (d), 131.9 (s), 137.8 (s) and 153.0 (s, C-3), C=O not seen; MS *m/z* (FAB+) 329.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 329.2124, $C_{21}H_{29}O_3$ requires 329.2117. HPLC (MeOH/H_2O, 90:10, λ_{max} = 279.3 nm) Rt = 2.37 min, 98%. Found: C, 76.60; H, 8.62. $C_{21}H_{28}O_3$ requires: C, 76.79; H, 8.59%.

16-Methylene-estradiol (65)

$NaBH_4$ (33 mg, 867 μmol) in H_2O (2 mL) was added dropwise to a stirred solution of **63** (50 mg, 177 μmol) in a mixture of MeOH/THF (3:1, 8 mL) at 0°C. The resulting colourless solution was stirred at 0°C for 10 minutes, after which AcOH glacial (4 drops) was added, followed by 10% aq. NaCl (10 mL). The white precipitate that formed was filtered, dried (38 mg) and recrystallised from acetone/hexane (1:3) to give **65** as white crystals (24 mg, 48%): mp 199-201°C [lit.[202] (acetone/hexane) 208-210°C]; IR (KBr) 3415 (OH), 3220 (br, OH), 2935-2865 (aliph CH), 1610-1445 (arom C=C and exocyclic C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.59 (3H, s, C-18-H_3), 1.19-2.38 (11H, m), 2.66-2.74 (2H, m, C-6-H_2), 3.79-3.81 (1H, m, C-17-H), 4.90-4.92 (2H, m, 1H exchanged with D_2O, C-1′-H and C-17-OH), 5.02-5.04 (1H, m, C-1′-H), 6.43 (1H, d, J = 2.6 Hz, C-4-H), 6.50 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H) and 7.04 (1H, d, J = 8.5 Hz, C-1-H), 9.00 (1H, s, exchanged with D_2O, C-3-OH); MS *m/z* (FAB+) 373.2 [82], 284.1 [100, M^+], 267.1 [47, $(M+H-OH)^+$]; Acc MS *m/z* (FAB+) 284.1778, $C_{19}H_{24}O_2$ requires 284.1776.

16-isobutylidene-estrone (66)

A solution of E1 (420 mg, 1.55 mmol) in anhydrous THF (5 mL) was added dropwise to a stirred solution of LDA (2.47 mL of a 1.8 M solution in heptane/THF/ethyl benzene, 4.44 mmol) in anhydrous THF (2 mL) at -78°C, under an atmosphere of N_2. After 2 hours of stirring at -78°C, isobutyraldehyde (185 μL, 2.03 mmol, freshly distilled from Na_2SO_4) was added. The resulting mixture was stirred overnight, during which it was allowed to warm to room temperature. The

solvent was then removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (70 mL), washed with H_2O (20 mL) then brine (20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a white foam (602 mg). The crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 9:1) to give **66** as a white foam (401 mg, 79%). This compound was recrystallised from EtOAc/hexane, yielding white crystals (325 mg, 64%): mp 188-190°C; IR (KBr) 3370 (OH), 2930-2890 (aliph CH), 1710 (C=O), 1645-1445 (arom C=C and exocyclic C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.06 (3H, d, J = 6.6 Hz, C-3'-H_3), 1.04 (3H, d, J = 6.6 Hz, C-4'-H_3), 1.40-2.70 (12H, m), 2.84-2.90 (2H, m, C-6-H_2), 4.67 (1H, s, exchanged with D_2O, OH), 6.46 (1H, ddd, J = 9.8 Hz, J = 2.7 Hz, J = 1.9 Hz, C-1'-H), 6.59 (1H, d, J = 2.7 Hz, C-4-H), 6.64 (1H, dd, J = 8.3 Hz, J = 2.7 Hz, C-2-H) and 7.16 (1H, d, J = 8.3 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 15.0 (q, C-18), 22.3 (2×q, C-3' and C-4'), 26.4 (2×t), 27.2 (t), 29.7 (d), 29.9 (t), 32.0 (t), 38.3 (d), 44.4 (d), 48.3 (d), 48.8 (s, C-13), 113.1 (d), 115.5 (d), 126.7 (d), 132.2 (s), 134.7 (s), 138.1 (s), 144.2 (d, C-1'), 153.8 (s, C-3) and 210.0 (s, C=O); MS *m/z* (FAB+) 325.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 325.2166, $C_{22}H_{29}O_2$ requires 325.2167. Found: C, 81.30; H, 8.71. $C_{22}H_{28}O_2$ requires: C, 81.44; H, 8.70%.

16-isobutylidene-estradiol (67)

A solution of $NaBH_4$ (57 mg, 1.51 mmol) in H_2O (3 mL) was added dropwise to a stirred solution of **66** (100 mg, 308 μmol) in a mixture of MeOH/THF (2:1, 9 mL) at 0°C. The resulting solution was stirred at 0°C for 20 minutes, after which AcOH glacial (5 drops) was added, followed by 10% aq. NaCl (20 mL). The white precipitate that formed was filtered, dried (102 mg) and recrystallised from acetone/hexane to give **67** as white crystals (60 mg, 60%): mp 143-145°C; IR (KBr) 3435-3325 (OH), 2955-2865 (aliph CH), 1695-1500 (arom C=C and exocyclic C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.67 (3H, s, C-18-H_3), 0.96 (3H, d, J = 6.6 Hz, C-3'-H_3), 0.99 (3H, d, J = 6.6 Hz, C-4'-H_3), 1.22-2.02 (13H, m), 2.81-2.87 (2H, m, C-6-H_2), 3.91-3.98 (1H, m, C-17-H), 4.66 (1H, s, exchanged with D_2O, OH), 5.32 (1H, ddd, J = 9.4 Hz, J = 4.7 Hz, J = 2.3 Hz, C-1'-H), 6.56 (1H, d, J = 2.7 Hz, C-4-H), 6.63 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H) and 7.16 (1H, d, J = 8.6 Hz, C-1-H); MS *m/z*

(FAB+) 326.2 [95, M^+], 309.2 [100, $(M\text{-}OH)^+$], 283.1 [50]; Acc MS *m/z* (FAB+) 326.2252, $C_{22}H_{30}O_2$ requires 326.2246.

16-(2′,2′-Dimethyl-propylidene)-estrone (68) and 3-(1′-hydroxy-2′,2′-dimethyl-propoxy)-16-(1″-hydroxy-2″,2″-dimethyl-propyl)-estrone (69)

This was prepared according to the procedure used to synthesise **66**. E1 (500 mg, 1.85 mmol) reacted with LDA (2.47 mL of a 1.8 M solution in heptane/THF/ethyl benzene, 4.44 mmol) and 2,2-dimethyl-propionaldehyde (261 μL, 2.40 mmol, freshly distilled from Na_2SO_4) for 2 days. Purification of the crude mixture by flash chromatography ($CHCl_3$/EtOAc, 9:1 to 8:2, gradient) gave two products:

The less polar fraction gave **68** as a white crystalline solid (264 mg, 42%). This compound was recrystallised from EtOAc to give white crystals (113 mg, 21%): mp 256-258°C; IR (KBr) 3350 (OH), 2960-2860 (aliph CH), 1710 (C=O), 1640-1450 (exocyclic C=C and arom C=C), 1225 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.90 (3H, s, C-18-H_3), 1.17 (9H, s, $C(CH_3)_3$), 1.41-2.45 (11H, m), 2.83-2.91 (2H, m, C-6-H_2), 4.72 (1H, s, exchanged with D_2O, OH), 6.59 (1H, d, J = 2.7 Hz, C-4-H), 6.63-6.65 (2H, m, C-2-H and C-1′-H) and 7.16 (1H, d, J = 8.0 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 14.6 (q, C-18), 26.1 (t), 26.8 (t), 27.0 (t), 29.5 (t), 29.7 (3×q, $C(CH_3)_3$), 31.7 (t), 33.7 (s, $C(CH_3)_3$), 38.0 (d), 44.1 (d), 47.0 (s, C-13), 48.0 (d), 112.5 (d), 114.9 (d), 126.0 (d), 131.8 (s), 132.8 (s), 137.5 (s), 146.5 (d, C-1′), 153.1 (s, C-3) and 209.3 (s, C=O); MS *m/z* (FAB+) 677.5 [54, $(2M+H)^+$], 492.2 [24, $(M+H+NBA)^+$], 339.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 339.2326, $C_{23}H_{31}O_2$ requires 339.2324. HPLC (MeOH/H_2O, 80:20; λ_{max} = 233.3 nm) Rt = 4.25 min, 99%. Found: C, 81.50; H, 8.96. $C_{23}H_{30}O_2$ requires: C, 81.61; H, 8.93%.

The more polar fraction gave **69** as a white solid (136 mg, 17%). An analytical sample was recrystallised from EtOAc/Hexane (1:2): mp 126-128°C; IR (KBr) 3445 (br, OH), 2970-2870 (aliph CH), 1700 (C=O), 1615-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.80 (3H, s, C-18-H_3), 0.93 (9H, s, $C(CH_3)_3$), 0.98-2.29 (13H, m), 1.25 (9H, s, $C(CH_3)_3$), 2.78-2.85 (2H, m, C-6-H_2), 3.23 (1H, dd, exchanged to a doublet with D_2O, J = 8.8 Hz, J = 4.9 Hz, C-1″-H), 4.68 (1H, s, exchanged with D_2O, OH), 4.97 (1H, s, C-1′-H), 6.55 (1H, d, J = 2.7 Hz, C-4-H), 6.62 (1H, dd, J = 8.4 Hz,

J = 2.7 Hz, C-2-H) and 7.13 (1H, d, J = 8.4 Hz, C-1-H), OH not seen; MS *m/z* (FAB+) 442.4 [100, $(M+H)^+$], 341.3 [98], 323.3 [34], 283.2 [38], 159.1 [30].

16-(2′,2′-Dimethyl-propylidene)-estradiol (70)

A solution of $NaBH_4$ (44 mg, 1.16 mmol) in H_2O (2.5 mL) was added to a stirred solution of **68** in a mixture of MeOH/THF (2:1, 15 mL) at 0°C. The colourless solution was stirred for 1 hour at 0°C, after which AcOH glacial (4 drops) was added, followed by 10% aq. NaCl solution (15 mL). The resulting white precipitate was filtered, dried (79 mg) and recrystallised from EtOAc/Hexane (1:8) to give **70** as white crystals (63 mg, 79%): mp 219-222°C; IR (KBr) 3550, 3410 (OH), 2960-2875 (aliph CH), 1610, 1505 (aliph C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.65 (3H, s, C-18-H_3), 1.10 (9H, s, $C(CH_3)_3$), 1.18-2.59 (12H, m), 2.82-2.88 (2H, m, C-6-H_2), 3.85-3.90 (1H, app d, J = 9.4 Hz, C-17-H), 4.60 (1H, s, exchanged with D_2O, OH), 5.46 (1H, dd, J = 4.9 Hz, J = 2.5 Hz, C-1′-H), 6.55 (1H, d, J = 2.8 Hz, C-4-H), 6.61 (1H, dd, J = 8.5 Hz, J = 2.8 Hz, C-2-H) and 7.14 (1H, d, J = 8.5 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 11.4 (q, C-18), 26.8 (t), 27.8 (t), 29.1 (t), 30.0 (t), 30.8 (3×q, $C(CH_3)_3$), 33.5 (s, $C(CH_3)_3$), 36.8 (t), 38.7 (d), 42.9 (s, C-13), 44.4 (d), 47.5 (d), 85.0 (d, C-17), 113.0 (d), 115.5 (d), 126.7 (d), 132.7 (s), 133.7 (d, C-1′), 138.3 (s), 140.2 (s) and 153.6 (s, C-3); MS *m/z* (FAB+) 340.2 [49, M^+], 323.2 [100, $(M-OH)^+$], 283.2 [92, $(M-C(CH_3)_3)^+$] ; Acc MS *m/z* (FAB+) 340.2390, $C_{23}H_{32}O_2$ requires 340.2402. HPLC (MeOH/H_2O, 96:4, λ_{max} = 280.5 nm) Rt = 2.54 min, 99.5%. Found: C, 81.00; H, 9.54. $C_{23}H_{32}O_2$ requires: C, 81.13; H, 9.47%.

16-(1′-Hydroxy-propyl)-estrone (71)

Following the procedure used to synthesise **66**, E1 (500 mg, 1.85 mmol) reacted with LDA (2.47 mL of a 1.8 M solution in heptane/THF/ethyl benzene, 4.44 mmol) and propionaldehyde (160 µL, 2.40 mmol, dried on $CaCl_2$ and freshly distilled from Na_2SO_4) overnight. Purification of the crude mixture by flash chromatography ($CHCl_3$/EtOAc, 8:2) gave **71** as a white solid (363 mg, 60%). This compound was recrystallised from EtOAc to give white crystals (49 mg) and a further crop of the product (170 mg) was obtained from the residue of the mother liquor upon recrystallisation from EtOAc/hexane (overall yield 36%): mp 248-252°C; IR (KBr) 3585 (OH), 3380 (br, OH), 2970-2835 (aliph CH), 1720 (C=O), 1600-1445 (arom

C=C), 1225 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.94 (3H, s, C-18-H_3), 1.01 (3H, t, *J* = 7.5 Hz, C-3'-H_3), 1.32-2.44 (14H, m), 2.83-2.91 (2H, m, C-6-H_2), 3.65-3.72 (1H, m, C-1'-H), 3.99 (1H, s, exchanged with D_2O, OH), 4.69 (1H, s, exchanged with D_2O, OH), 6.59 (1H, d, *J* = 2.7 Hz, C-4-H), 6.65 (1H, dd, *J* = 8.4 Hz, *J* = 2.7 Hz, C-2-H) and 7.15 (1H, d, *J* = 8.4 Hz, C-1-H); MS *m/z* (FAB+) 329.2 [100, $(M+H)^+$], 311.2 [30, $(M-OH)^+$]; Acc MS *m/z* (FAB+) 329.2119, $C_{21}H_{29}O_3$ requires 329.2117. Found: C, 76.60; H, 8.62. $C_{21}H_{28}O_3$ requires: C, 76.79; H, 8.59%.

5.4.5 D-ring fused pyrazole derivatives of E1 and precursors

3-Benzyloxy-16-hydroxymethylene-estra-1,3,5(10)-triene-17-one (72)

^{t}BuOK (9.4 g, 84.1 mmol) was added portionwise to a stirred solution of **3** (10 g, 27.7 mmol) in anhydrous toluene (250 mL) at room temperature, under an atmosphere of N_2. After stirring for 20 minutes, ethyl formate (14.8 mL, 194 mmol) was added and the resulting suspension stirred for 2.5 hours. The final mixture was poured into H_2O (300 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×200 mL), washed with H_2O (3×100 mL), then brine (2×100 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was triturated with boiling EtOAc to give **72** as an off-white solid (9.25 g, 86%): mp 149-151°C; IR (KBr) 3235 (OH), 2930-2855 (aliph CH), 1700, 1675-1500 (C=O, C=C and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.81 (3H, s, C-18-H_3), 1.31-2.62 (11H, m), 2.79-2.85 (2H, m, C-6-H_2), 5.05 (2H, s, OCH_2Ar), 6.71 (1H, d, *J* = 2.7 Hz, C-4-H), 6.75 (1H, dd, *J* = 8.4 Hz, *J* = 2.7 Hz, C-2-H), 7.16 (1H, d, *J* = 8.4 Hz, C-1-H), 7.28-7.33 (1H, m, =C*H*OH), 7.35-7.43 (5H, m, C_6H_5) and 10.6-10.9 (1H, br s, exchanged with D_2O, =CHO*H*); δ_C (DMSO-d_6, 100.4 MHz) 15.3 (q, C-18), 24.9 (t), 26.5 (t), 27.1 (t), 30.0 (t), 32.3 (t), 38.2 (d), 44.3 (d), 48.6 (s, C-13), 49.1 (d), 69.7 (t, OCH_2Ar), 112.9 (d), 113.9 (s, C-16), 115.2 (d), 126.7 (d), 128.1 (2×d), 128.3 (d), 129.0 (2×d), 132.7 (s), 138.0 (2×s), 150.8 (d, C-1'), 156.7 (s, C-3) and 209.1 (s, C=O); MS *m/z* (FAB+) 389.3 [31, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 389.2099, $C_{26}H_{29}O_3$ requires 389.2117.

3-Benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-pyrazole (73)

Hydrazine hydrate (751 μL, 15.44 mmol) was added to a suspension of **72** (4 g, 10.3 mmol) in EtOH (200 mL) at room temperature, under an atmosphere of N_2. The resulting yellow suspension was heated to reflux for 45 minutes and allowed to cool down. After acidification with 5M HCl, the solvent was removed under reduced pressure until precipitation of the product. H_2O (20 mL) was then added and the precipitate was filtered and dried to give **73** as an off-white powder (3.73 g, 94%): mp 102-105°C; IR (KBr) 3410 (NH), 2930-2860 (aliph CH), 1610-1500 (C=N and arom C=C), 1255 (C-O) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.91 (3H, s, C-18-H_3), 1.36-2.58 (11H, m), 2.78-2.87 (2H, m, C-6-H_2), 5.05 (2H, s, OCH_2Ar), 6.72 (1H, d, J = 2.7 Hz, C-4-H), 6.76 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.18 (1H, d, J = 8.6 Hz, C-1-H), 7.27 (1H, s, C-5'-H), 7.29-7.44 (5H, m, C_6H_5) and 12.00 (1H, s, exchanged with D_2O, NH); MS *m/z* (FAB+) 385.3 [75, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 385.2280, $C_{26}H_{29}N_2O$ requires 385.2280.

3-Benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(1'-methyl)-pyrazole (74) and 3-benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(2'-methyl)-pyrazole (75)

Following method 5, **73** (150 mg, 390 μmol) was treated with NaH (19 mg, 468 μmol) in DMF (6 mL) and the subsequent reaction with methyl iodide (57 μL, 780 μmol) was complete within 50 minutes. Purification of the crude mixture by flash chromatography (DCM/EtOAc, 98:2 to 95:5, gradient) gave two products:

The less polar fraction gave **74** as a light yellow oil that crystallised on standing (52 mg, 33%): mp 133-135°C; IR (KBr) 2930-2860 (aliph CH), 1606-1500 (C=N and arom C=C), 1245 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.42-2.67 (11H, m), 2.81-2.99 (2H, m, C-6-H_2), 3.84 (3H, s, N-CH_3), 5.03 (2H, s, OCH_2Ar), 6.73 (1H, d, J = 2.5 Hz, C-4-H), 6.79 (1H, dd, J = 8.5 Hz, J = 2.5 Hz, C-2-H), 6.97 (1H, s, C-5'-H), 7.22 (1H, d, J = 8.5 Hz, C-1-H) and 7.29-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 18.4 (q, C-18), 24.0 (t), 26.2 (t), 27.4 (t), 29.6 (t), 34.1 (t), 37.5 (d), 38.5 (q, N-CH_3), 40.8 (s, C-13), 44.4 (d), 61.1 (d), 69.8 (t, OCH_2Ar), 111.9 (d), 114.6 (d), 121.5 (s), 124.0 (d), 125.9 (d), 127.1 (2×d), 127.5 (d), 128.2 (2×d), 132.6 (s), 136.9 (s), 137.5 (s), 156.4 (s) and 168.6 (s); MS *m/z* (FAB+) 399.3 [74, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$], 73.0 [24]; Acc MS *m/z* (FAB+) 399.2447,

$C_{27}H_{31}N_2O$ requires 399.2436. Found: C, 81.20; H, 7.55; N, 7.05. $C_{27}H_{30}N_2O$ requires: C, 81.37; H, 7.59; N, 7.03%.

The more polar fraction gave **75** as an off-white solid (55 mg, 35%): mp 168-171°C; IR (KBr) 2930-2870 (aliph CH), 1640-1495 (C=N and arom C=C), 1255 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.44-2.64 (11H, m), 2.85-2.98 (2H, m, C-6-H_2), 3.82 (3H, s, N-CH_3), 5.04 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.8 Hz, C-4-H), 6.80 (1H, dd, J = 8.6 Hz, J = 2.8 Hz, C-2-H), 7.15 (1H, s, C-5'-H), 7.20 (1H, d, J = 8.6 Hz, C-1-H) and 7.30-7.45 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 17.9 (q, C-18), 24.6 (t), 26.5 (t), 27.7 (t), 30.1 (t), 34.5 (t), 37.3 (q, N-CH_3), 37.8 (d), 42.0 (s, C-13), 44.6 (d), 62.3 (d), 70.3 (t, OCH_2Ar), 112.6 (d), 115.1 (d), 123.9 (s), 126.2 (d), 127.6 (2×d), 128.1 (d), 128.7 (2×d), 132.7 (s), 133.4 (d), 137.4 (s), 138.1 (s) and 157.0 (s), 1 singlet not seen; MS *m/z* (FAB+) 399.3 [55, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 399.2434, $C_{27}H_{31}N_2O$ requires 399.2436. Found: C, 81.10; H, 7.53; N, 6.87. $C_{27}H_{30}N_2O$ requires: C, 81.37; H, 7.59; N, 7.03%.

3-Benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(1'-isobutyl)-pyrazole (76) and 3-benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(2'-isobutyl)-pyrazole (77)

Following method 5, **73** (250 mg, 650 μmol) was treated with NaH (31 mg, 780 μmol) in DMF (10 ml) and the subsequent reaction with 1-bromo-2-methyl-propane (124 μL, 1.30 mmol) was complete within 2 hours. Purification of the crude mixture by flash chromatography (DCM/EtOAc, 95:5) gave two products:

The less polar fraction gave **76** as a pale yellow oil (121 mg, 42%): IR (KBr) 2960-2870 (aliph CH), 1705, 1640-1495 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.87 (3H, d, J = 6.6 Hz, C-3''-H_3), 0.89 (3H, d, J = 6.6 Hz, C-4''-H_3), 1.01 (3H, s, C-18-H_3), 1.42-2.98 (14H, m), 3.81 (1H, dd, J_{BA} = 13.8 Hz, J = 7.5 Hz, N-C$H_A$$H_B$), 3.86 (1H, dd, J_{AB} = 13.8 Hz, J = 7.5 Hz, N-C$H_A$$H_B$), 5.04 (2H, s, OCH_2Ar), 6.71-6.75 (1H, m, C-4-H), 6.79 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 6.94-6.99 (1H, m, C-5'-H), 7.22 (1H, d, J = 8.6 Hz, C-1-H) and 7.31-7.46 (5H, m, C_6H_5); MS *m/z* (FAB+) 441.2 [100, $(M+H)^+$], 91.1 [93, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 441.2894, $C_{30}H_{37}N_2O$ requires 441.2906.

The more polar fraction gave **77** as a white crystalline solid (56 mg, 19%): mp 126-128°C; IR (KBr) 2960-2870 (aliph CH), 1635-1455 (C=N and arom C=C), 1255 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, d, J = 6.6 Hz, C-3″-H_3), 0.95 (3H, d, J = 6.6 Hz, C-4″-H_3), 1.02 (3H, s, C-18-H_3), 1.46-2.60 (12H, m), 2.89-2.94 (2H, m, C-6-H_2), 3.74 (1H, dd, J_{BA} = 13.3 Hz, J = 7.8 Hz, N-C$H_A$$H_B$), 3.85 (1H, dd, J_{AB} = 13.3 Hz, J = 7.4 Hz, N-C$H_A$$H_B$), 5.04 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.6 Hz, C-4-H), 6.79 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.17-7.21 (2H, m, C-1-H and C-5′-H) and 7.31-7.45 (5H, m, C_6H_5); MS *m/z* (FAB+) 441.1 [100, $(M+H)^+$], 91.1 [60, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 441.2898, $C_{30}H_{37}N_2O$ requires 441.2906.

3-Benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(1′-methylacetate)-pyrazole (78) and 3-benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(2′-methylacetate)-pyrazole (79)
Following method 5, **73** (300 mg, 78 μmol) was treated with NaH (47 mg, 1.17 mmol) in DMF (10 mL) and the subsequent reaction with methyl chloroacetate (136 μL, 1.56 mmol) was complete within 2.5 hours. Purification of the crude mixture by flash chromatography (DCM to DCM/EtOAc, 8:2, gradient, flashmaster) gave two products:

The less polar fraction gave **78** as a white crystalline solid (173 mg, 48%): mp 133-135°C; IR (KBr) 2960-2850 (aliph CH), 1750, 1740 (C=O), 1615-1500 (C=N and arom C=C), 1265, 1255 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.03 (3H, s, C-18-H_3), 1.44-2.69 (11H, m), 2.85-2.99 (2H, m, C-6-H_2), 3.76 (3H, s, OCH_3), 4.83 (1H, d, J_{BA} = 17.8 Hz, N-C$H_A$$CH_B$), 4.88 (1H, d, J_{AB} = 17.8 Hz, N-CH_ACH_B), 5.04 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.6 Hz, C-4-H), 6.79 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.07 (1H, s, C-5′-H), 7.22 (1H, d, J = 8.6 Hz, C-1-H) and 7.29-7.44 (5H, m, C_6H_5); δ_C ($CDCl_3$, 100.4 MHz) 18.9 (q, C-18), 24.5 (t), 26.7 (t), 27.9 (t), 30.1 (t), 34.4 (t), 38.0 (d), 41.4 (s, C-13), 44.8 (d), 52.9 (d or q), 53.2 (t, N-CH_2), 61.3 (d or q), 70.3 (t, OCH_2Ar), 112.5 (d), 115.1 (d), 123.2 (s), 125.0 (d), 126.4 (d), 127.7 (2×d), 128.1 (d), 128.7 (2×d), 133.1 (s), 137.5 (s), 138.0 (s), 156.9 (s), 169.1 (s) and 169.8 (s); MS *m/z* (FAB+) 457.3 [55, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$], 73.0 [72]; Acc MS *m/z* (FAB+) 457.2495, $C_{29}H_{33}N_2O_3$ requires 457.2491.

The more polar fraction gave **79** as a pale yellow solid (70 mg, 20%): mp 78-82°C; IR (KBr) 2930-2850 (aliph CH), 1760, 1740 (C=O), 1635-1500 (C=N and arom C=C), 1260, 1210 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.42-2.64 (11H, m), 2.86-2.98 (2H, m, C-6-H_2), 3.78 (3H, s, OCH_3), 4.80 (1H, d, J_{BA} = 17.2 Hz, N-CH_ACH_B), 4.86 (1H, d, J_{AB} = 17.2 Hz, N-CH_ACH_B), 5.03 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.6 Hz, C-4-H), 6.78 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.18 (1H, d, J = 8.6 Hz, C-1-H), 7.25 (1H, s, C-5′-H) and 7.31-7.44 (5H, m, C_6H_5); MS *m/z* (FAB+) 457.3 [64, $(M+H)^+$], 91.1 [100, $(CH_2Ar)^+$], 73.0 [27]; Acc MS *m/z* (FAB+) 457.2507, $C_{29}H_{33}N_2O_3$ requires 457.2491.

3-Benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(1′-methoxyethyl)-pyrazole (80) and 3-benzyloxy-estra-1,3,5(10)-triene-[17,16-c]-(2′-methoxyethyl)-pyrazole (81)

Following method 5, **73** (300 mg, 780 μmol) was treated with NaH (47 mg, 1.17 mmol) in DMF (10 mL) and the subsequent reaction with 1-chloro-2-methoxy-ethane (142 μL, 1.56 mmol) was complete within 4 hours. Purification of the crude mixture by flash chromatography (DCM/EtOAc, 9:1) gave two products:

The less polar fraction gave **80** as a pale yellow solid (92 mg, 27%): mp 97-98°C; IR (KBr) 2930-2870 (aliph CH), 1615, 1500 (C=N and arom C=C), 1255, 1105 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.46-2.66 (11H, m), 2.84-2.99 (2H, m, C-6-H_2), 3.33 (3H, s, OCH_3), 3.67-3.76 (2H, m, C-1″-H_2 or C-2″-H_2), 4.17-4.28 (2H, m, C-1″-H_2 or C-2″-H_2), 5.04 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.8 Hz, C-4-H), 6.80 (1H, dd, J = 8.3 Hz, J = 2.8 Hz, C-2-H), 7.09 (1H, s, C-5′-H), 7.23 (1H, d, J = 8.3 Hz, C-1-H) and 7.31-7.45 (5H, m, C_6H_5); MS *m/z* (FAB+) 443.3 [100, $(M+H)^+$], 91.1 [80, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 443.2696, $C_{29}H_{35}N_2O_2$ requires 443.2698.

The more polar fraction gave **81** as a pale yellow solid (80 mg, 23%): mp 86-88°C; IR (KBr) 2930-2850 (aliph CH), 1635-1495 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.04 (3H, s, C-18-H_3), 1.42-2.66 (11H, m), 2.84-2.99 (2H, m, C-6-H_2), 3.32 (3H, s, OCH_3), 3.73-3.83 (2H, m, C-1″-H_2 or C-2″-H_2), 4.14-4.22 (2H, m, C-1″-H_2 or C-2″-H_2), 5.04 (2H, s, OCH_2Ar), 6.74 (1H, d, J = 2.7 Hz, C-4-H), 6.79 (1H, dd,

J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.19-7.21 (2H, m, C-1-H and C-5′-H) and 7.30-7.44 (5H, m, C_6H_5); MS *m/z* (FAB+) 443.3 [100, $(M+H)^+$], 91.1 [95, $(CH_2Ar)^+$], 73.1 [44]; Acc MS *m/z* (FAB+) 443.2712, $C_{29}H_{35}N_2O_2$ requires 443.2698.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(1′-methyl)-pyrazole (82)

Following method 2, a suspension of **74** (50 mg, 125 μmol) and Pd-C (10%, 20 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give a pale yellow solid (31 mg). The crude product was recrystallised from EtOH/H_2O to yield **82** as off-white crystals (26 mg, 68%): mp 287-289°C; IR (KBr) 3115 (OH or arom CH), 2975-2855 (aliph CH), 1605-1500 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.89 (3H, s, C-18-H_3), 1.32-2.57 (11H, m), 2.69-2.83 (2H, m, C-6-H_2), 3.73 (3H, s, N-CH_3), 6.45 (1H, d, J = 2.4 Hz, C-4-H), 6.52 (1H, dd, J = 8.5 Hz, J = 2.4 Hz, C-2-H), 7.06 (1H, d, J = 8.5 Hz, C-1-H), 7.24 (1H, s, C-5′-H) and 9.02 (1H, s, OH); MS *m/z* (FAB+) 309.2 [100, $(M+H)^+$], 219.2 [52]; Acc MS *m/z* (FAB+) 309.1975, $C_{20}H_{25}N_2O$ requires 309.1967. HPLC (MeOH/H_2O, 96:4, λ_{max} = 280.5 nm) Rt = 1.90 min, 98%. Found: C, 77.80; H, 7.87; N, 8.90. $C_{20}H_{24}N_2O$ requires: C, 77.89; H, 7.84; N, 9.08%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(2′-methyl)-pyrazole (83)

Following method 2, a suspension of **75** (50 mg, 125 μmol) and Pd-C (10%, 20 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give a white solid (40 mg). The crude product was recrystallised from EtOH to yield **83** as white crystals (20 mg, 53%): mp 321-323°C (dec.); IR (KBr) 3105-3010 (arom CH), 2935-2860 (aliph CH), 1610-1500 (C=N and arom C=C); δ_H (DMSO-d_6, 400 MHz) 0.95 (3H, s, C-18-H_3), 1.32-2.52 (11H, m), 2.69-2.83 (2H, m, C-6-H_2), 3.73 (3H, s, N-CH_3), 6.45 (1H, d, J = 2.6 Hz, C-4-H), 6.52 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H), 7.04 (1H, s, C-5′-H), 7.06 (1H, d, J = 8.4 Hz, C-1-H) and 9.03 (1H, s, OH); MS *m/z* (FAB+) 391.3 [28], 309.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 309.1974, $C_{20}H_{25}N_2O$ requires 309.1967. HPLC (MeOH/H_2O, 96:4, λ_{max} = 280.5 nm) Rt = 2.41 min, 99%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(1′-isobutyl)-pyrazole (84)

Following method 2, a suspension of **76** (110 mg, 250 μmol) and Pd-C (10%, 40 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give a white solid (78

mg). The crude product was recrystallised from MeOH to yield **84** as colourless crystals (42 mg, 48%): mp 122-123°C; IR (KBr) 3235 (OH), 2955-2853 (aliph CH), 1635, 1610, 1575-1455 (C=N and arom C=C), 1230 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.87 (3H, d, J = 6.4 Hz, C-3″-H_3), 0.89 (3H, d, J = 6.4 Hz, C-4″-H_3), 1.01 (3H, s, C-18-H_3), 1.42-2.65 (12H, m), 2.81-2.95 (2H, m, C-6-H_2), 3.81 (1H, dd, J_{BA} = 13.7 Hz, J = 7.4 Hz, N-C$H_A$$H_B$), 3.86 (1H, dd, J_{AB} = 13.7 Hz, J = 7.4 Hz, N-C$H_A$$H_B$), 5.61 (1H, s, exchanged with D_2O, OH), 6.59 (1H, d, J = 2.6 Hz, C-4-H), 6.65 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H), 6.97 (1H, s, C-5′-H) and 7.16 (1H, d, J = 8.4 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 18.5 (q, C-18), 20.0 (2×t, C-3″ and C-4″), 24.1 (t), 26.4 (t), 27.5 (t), 29.6 (t), 30.0 (d), 34.2 (t), 37.6 (d), 41.0 (s, C-13), 44.5 (d), 59.6 (t, N-CH_2), 61.2 (d), 112.8 (d), 115.3 (d), 121.2 (s), 123.9 (d), 126.4 (d), 132.5 (s), 138.1 (s), 153.8 (s) and 168.7 (s); MS *m/z* (FAB+) 351.3 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 351.2448, $C_{23}H_{31}N_2O$ requires 351.2436. HPLC (MeOH/H_2O, 80:20; λ_{max} = 279.3 nm) Rt = 3.27 min, 99%. Found: C, 75.20; H, 8.79; N, 7.42. $C_{23}H_{30}N_2O$.MeOH requires: C, 75.35; H, 8.96; N, 7.32%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(2′-isobutyl)-pyrazole (85)

Following method 2, a suspension of **77** (45 mg, 102 μmol) and Pd-C (10%, 20 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give a light yellow solid (103 mg). The crude product was recrystallised from EtOAc/hexane to yield **85** as off-white crystals (33 mg, 92%): mp 194-196°C (dec.); IR (KBr) 3195 (OH), 2960-2845 (aliph CH), 1620, 1585, 1500 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, d, J = 6.6 Hz, C-3″-H_3), 0.95 (3H, d, J = 6.6 Hz, C-4″-H_3), 1.03 (3H, s, C-18-H_3), 1.41-2.63 (12H, m), 2.84-2.91 (2H, m, C-6-H_2), 3.77 (1H, dd, J_{BA} = 13.4 Hz, J = 8.2 Hz, N-C$H_A$$H_B$), 3.89 (1H, dd, J_{AB} = 13.4 Hz, J = 7.6 Hz, N-C$H_A$$H_B$), ~5.50 (~1H, br s, exchanged with D_2O, OH), 6.60 (1H, d, J = 2.6 Hz, C-4-H), 6.66 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H), 7.14 (1H, d, J = 8.4 Hz, C-1-H) and 7.22 (1H, s, C-5′-H); MS *m/z* (FAB+) 351.3 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 351.2444, $C_{23}H_{31}N_2O$ requires 351.2436. HPLC (MeOH/H_2O, 80:20; λ_{max} = 279.3 nm) Rt = 3.59 min, 100%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(1'-methylacetate)-pyrazole (86)

Following method 2, a suspension of **78** (90 mg, 197 μmol) and Pd-C (10%, 40 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 6 hours to give a white solid (66 mg). The crude product was recrystallised from MeOH to yield **86** as colourless crystals (49 mg, 68%): mp 236-239°C; IR (KBr) 3210 (OH), 2955-2855 (aliph CH), 1755 (C=O), 1610-1455 (C=N and arom C=C), 1215 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.03 (3H, s, C-18-H_3), 1.41-2.69 (11H, m), 2.81-2.96 (2H, m, C-6-H_2), 3.75 (3H, s, OCH_3), 4.83 (1H, d, J_{BA} = 17.6 Hz, N-C$H_A$$H_B$), 4.88 (1H, d, J_{AB} = 17.6 Hz, N-C$H_A$$H_B$), 5.04 (1H, s, OH), 6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.64 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.07 (1H, s, C-5'-H), 7.16 (1H, d, J = 8.4 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 18.8 (q, C-18), 24.5 (t), 26.7 (t), 27.9 (t), 29.9 (t), 34.3 (t), 38.0 (d), 41.4 (s, C-13), 44.8 (d), 52.9 (d or q), 53.1 (t, N-CH_2), 61.3 (d or q), 113.0 (d), 115.5 (d), 123.3 (s), 125.2 (d), 126.5 (d), 132.5 (s), 138.1 (s), 153.9 (s), 169.1 (s) and 169.8 (s); MS *m/z* (FAB+) 367.3 [100, $(M+H)^+$], 73.1 [29]; Acc MS *m/z* (FAB+) 367.2038, $C_{22}H_{27}N_2O_3$ requires 367.2022. Found: C, 69.60; H, 7.47; N, 7.17. $C_{22}H_{26}N_2O_3$.MeOH requires: C, 69.32; H, 7.59; N, 7.03%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(2'-methylacetate)-pyrazole (87)

Following method 2, a suspension of **79** (60 mg, 131 μmol) and Pd-C (10%, 25 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give a light brown foam (34 mg). The crude product was purified by flash chromatography (DCM/EtOAc, 8 :2) to yield **87** as a light yellow foam (18 mg, 37%): mp 92-95°C; IR (KBr) 3250 (OH), 2925-2855 (aliph CH), 1760, 1745 (C=O), 1615-1455 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.42-2.64 (12H, m), 2.84-2.91 (2H, m, C-6-H_2), 3.77 (3H, s, OCH_3), 4.81 (1H, d, J_{BA} = 17.4 Hz, N-C$H_A$$H_B$), 4.87 (1H, d, J_{AB} = 17.4 Hz, N-C$H_A$$H_B$), 6.59 (1H, d, J = 2.7 Hz, C-4-H), 6.64 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.12 (1H, d, J = 8.6 Hz, C-1-H) and ~7.25 (~1H, s, C-5'-H, under solvent peak); MS *m/z* (FAB+) 367.1 [37, $(M+H)^+$], 97.0 [39], 73.0 [55], 57.0 [100]; Acc MS *m/z* (FAB+) 367.2029, $C_{22}H_{27}N_2O_3$ requires 367.2022.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(1'-methoxyethyl)-pyrazole (88)

Following method 2, a suspension of **80** (80 mg, 181 μmol) and Pd-C (10%, 35 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 6 hours to give a white crystalline

solid (58 mg). The crude product was recrystallised from MeOH to yield **88** as white crystals (32 mg, 50%): mp 123-125°C; IR (KBr) 3235 (OH), 2930-2850 (aliph CH), 1640-1505 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.43-2.66 (11H, m), 2.81-2.93 (2H, m, C-6-H_2), 3.33 (3H, s, OCH_3), 3.67-3.75 (2H, m, C-1″-H_2 or C-2″-H_2), 4.17-4.28 (2H, m, C-1″-H_2 or C-2″-H_2), 5.57 (1H, br s, exchanged with D_2O, OH), 6.59 (1H, d, *J* = 2.7 Hz, C-4-H), 6.64 (1H, dd, *J* = 8.4 Hz, *J* = 2.7 Hz, C-2-H), 7.09 (1H, s, C-5′-H) and 7.16 (1H, d, *J* = 8.4 Hz, C-1-H); MS *m/z* (FAB+) 353.3 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 353.2232, $C_{22}H_{29}N_2O_2$ requires 353.2229. HPLC (MeOH/H_2O, 80:20, λ_{max} = 280.5 nm) Rt = 2.47 min, 98%. Found: C, 73.20; H, 8.07; N, 7.65. $C_{22}H_{28}N_2O_2.(MeOH)_{1/2}$ requires: C, 73.34; H, 8.21; N, 7.60%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(2′-methoxyethyl)-pyrazole (89)

Following method 2, a suspension of **81** (70 mg, 158 μmol) and Pd-C (10%, 30 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated overnight to give an off-white foam (50 mg). The crude product was purified by flash chromatography (DCM/EtOAc, 8:2) to yield **89** as an off-white oil (25 mg, 45%): IR (KBr) 2920-2855 (aliph CH), 1610-1500 (C=N and arom C=C), 1255 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.05 (3H, s, C-18-H_3), 1.42-2.61 (11H, m), 2.82-2.95 (2H, m, C-6-H_2), 3.31 (3H, s, OCH_3), 3.74-3.82 (2H, m, C-1″-H_2 or C-2″-H_2), 4.12-4.23 (2H, m, C-1″-H_2 or C-2″-H_2), 6.60 (1H, d, *J* = 2.6 Hz, C-4-H), 6.66 (1H, dd, *J* = 8.6 Hz, *J* = 2.6 Hz, C-2-H), 7.14 (1H, d, *J* = 8.6 Hz, C-1-H) and 7.23 (1H, s, C-5′-H), OH not seen; MS *m/z* (FAB+) 353.3 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 353.2236, $C_{22}H_{29}N_2O_2$ requires 353.2229.

3-*O*-*tert*-Butyl-dimethylsilyl-estrone (90)

Imidazole (6.3 g, 92.4 mmol) and *tert*-butyl-dimethylsilyl chloride (7.8 g, 51.8 mmol) were added to a stirred solution of E1 (10.0 g, 37.0 mmol) in anhydrous DMF (300 mL) at room temperature, under an atmosphere of N_2. After stirring overnight, the resulting white suspension was poured into H_2O (500 mL). The organics were extracted with EtOAc (500 mL + 300 mL), washed with H_2O (2×200 mL), then brine (2×200 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was recrystallised from EtOH to give **90** as white needles (14.0 g,

98%): mp 171-173°C [lit.[232] (EtOH) 170-172°C]; IR (KBr) 2960-2855 (aliph CH), 1730 (C=O), 1605, 1495 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.19 (6H, s, $Si(CH_3)_2$), 0.91 (3H, s, C-18-H_3), 0.98 (9H, s, $C(CH_3)_3$), 1.37-2.55 (13H, m), 2.81-2.89 (2H, m, C-6-H_2), 6.57 (1H, d, J = 2.6 Hz, C-4-H), 6.62 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H) and 7.12 (1H, d, J = 8.3 Hz, C-1-H); MS *m/z* (FAB+) 384.2 [100, M^+], 327.2 [44, $(M\text{-}C(CH_3)_3)^+$], 73.0 [44]; Acc MS *m/z* (FAB+) 384.2477, $C_{24}H_{36}O_2Si$ requires 384.2485.

3-*O-tert*-Butyl-dimethylsilyl-16-hydroxymethylene-estrone (91)

NaOMe (4.8 g, 70.2 mmol) was added portionwise to a stirred solution of **90** (9 g, 23.4 mmol) in anhydrous toluene (250 mL) at room temperature, under an atmosphere of N_2. After stirring for 20 minutes, ethyl formate (13.2 mL, 164 mmol) was added and the resulting bright yellow solution was stirred overnight. The final thick yellow suspension was poured into H_2O (300 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×500 mL), washed with H_2O (3×200 mL), then brine (2×200 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give an off-white crude product (9.49 g, 98%). For analysis a sample was recrystallised from EtOH to give **91** as white crystals: mp 168-171°C; IR (KBr) 2930-2855 (aliph CH), 1710 (C=O), 1695-1500, 1495 (C=C and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.17 (6H, s, $Si(CH_3)_2$), 0.83 (3H, s, C-18-H_3), 0.96 (9H, s, $C(CH_3)_3$), 1.32-2.63 (11H, m), 2.77-2.85 (2H, m, C-6-H_2), 6.55 (1H, d, J = 2.6 Hz, C-4-H), 6.61 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H), 7.14 (1H, d, J = 8.3 Hz, C-1-H), 7.40 (1H, s, =C*H*OH) and 10.69 (1H, s, exchanged with D_2O, =CHO*H*); δ_C (DMSO-d_6,100.4 MHz) -4.3 (2×q, $Si(CH_3)_2$), 14.5 (q, C-18), 18.0 (s, $C(CH_3)_3$), 24.1 (t), 25.6 (t and 3×q, $C(CH_3)_3$), 26.2 (t), 29.0 (t), 31.5 (t), 37.3 (d), 43.6 (d), 47.7 (s, C-13), 48.4 (d), 113.0 (s, C-16), 116.9 (d), 119.4 (d), 125.9 (d), 132.5 (s), 137.3 (s), 149.9 (d, C-1′), 152.5 (s, C-3) and 208.2 (s, C=O); MS *m/z* (FAB+) 413.2 [68, $(M+H)^+$], 355.1 [33, $(M\text{-}C(CH_3)_3)^+$], 72.9 [100]; Acc MS *m/z* (FAB+) 413.2492, $C_{25}H_{37}O_3Si$ requires 413.2512. Found: C, 72.77; H, 8.79. $C_{25}H_{36}O_3Si$ requires: C, 72.40; H, 8.90%.

3-*O-tert*-Butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-c]-pyrazole (92)

Hydrazine hydrate (440 μL, 9.1 mmol) was added to a suspension of **91** (2.5 g, 6.1 mmol) in EtOH (125 mL) at room temperature, under an atmosphere of N_2. The resulting mixture was heated to reflux for 45 minutes. After cooling down, the solvent was removed under reduced pressure, H_2O (200 mL) added and the mixture acidified with 5M HCl. The organics were extracted with EtOAc (2×10 mL), washed with H_2O (2×50 mL), then brine (2×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a yellow foam (2.9 g). The crude product was recrystallised from EtOH/H_2O to give **92** as yellow crystals (2.0 g, 81%): mp 122-124°C; IR (KBr) 3190 (arom CH), 2930-2855 (aliph CH), 1605-1495 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.18 (6H, s, $Si(CH_3)_2$), 0.95 (3H, s, C-18-H_3), 0.96 (9H, s, $C(CH_3)_3$), 1.36-2.61 (11H, m), 2.79-2.87 (2H, m, C-6-H_2), 6.55 (1H, d, J = 2.3 Hz, C-4-H), 6.62 (1H, dd, J = 8.5 Hz, J = 2.3 Hz, C-2-H), 7.16 (1H, d, J = 8.5 Hz, C-1-H), 7.28 (1H, s, C-5′-H) and 12.02 (1H, s, exchanged with D_2O, NH); δ_C ($CDCl_3$, 100.4 MHz) -3.8 (2×q, $Si(CH_3)_2$), 18.6 (s, $C(CH_3)_3$), 18.8 (q, C-18), 24.3 (t), 26.2 (3×q, $C(CH_3)_3$), 26.6 (t), 27.9 (t), 30.0 (t), 34.5 (t), 38.0 (d), 41.1 (s, C-13), 44.9 (d), 61.9 (d), 117.4 (d), 120.2 (d), 121.8 (s), 123.2 (s), 126.2 (d), 133.2 (s), 137.8 (s), 153.5 (s) and 168.6 (s); MS *m/z* (FAB+) 409.2 [100, $(M+H)^+$], 73.0 [33]; Acc MS *m/z* (FAB+) 409.2662, $C_{25}H_{37}N_2OSi$ requires 409.2675. Found: C, 71.40; H, 8.93; N, 6.53. $C_{25}H_{36}N_2OSi.(H_2O)_{2/3}$ requires: C, 71.38; H, 8.95; N, 6.66%.

3-*O-tert*-Butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-c]-(1′-propionitrile)-pyrazole (93) and 3-*O-tert*-butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-c]-(2′-propionitrile)-pyrazole (94)

^{t}BuOK (91 mg, 807 μmol) was added to a stirred solution of **92** (300 mg, 734 μmol) in anhydrous THF (10 mL) at 0°C, under an atmosphere of N_2. After 20 minutes of stirring, acrylonitrile (58 μL, 881 μmol) was added and the resulting bright orange solution was stirred for 5 hours at room temperature. The resulting dark orange mixture was then concentrated under reduced pressure and H_2O (50 mL) was added. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced

pressure. The crude product was purified by flash chromatography (DCM/EtOAc, 9:1) to give two products:

The less polar fraction gave **93** as a light yellow oil (29 mg, 8%): IR (KBr) 2930-2860 (aliph CH), 2250 (CN), 1640-1495 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.20 (6H, s, $Si(CH_3)_2$), 0.98 (9H, s, $C(CH_3)_3$), 1.02 (3H, s, C-18-H_3), 1.42-2.67 (11H, m), 2.80-3.01 (4H, m, C-6-H_2 and CH_2CN), 4.28-4.40 (2H, m, N-CH_2), 6.57 (1H, d, J = 2.6 Hz, C-4-H), 6.63 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.10 (1H, s, C-5′-H) and 7.14 (1H, d, J = 8.5 Hz, C-1-H); MS *m/z* (FAB+) 462.2 [100, $(M+H)^+$], 73.0 [55]; Acc MS *m/z* (FAB+) 462.2914, $C_{28}H_{40}N_3OSi$ requires 462.2941.

The more polar fraction gave **94** as a light brown oil that crystallised on standing (42 mg, 12%): mp 80-82°C; IR (KBr) 2930-2855 (aliph CH), 2255 (CN), 1705-1445 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.20 (6H, s, $Si(CH_3)_2$), 0.98 (9H, s, $C(CH_3)_3$), 1.07 (3H, s, C-18-H_3), 1.41-2.68 (11H, m), 2.81-3.06 (4H, m, C-6-H_2 and CH_2CN), 4.21-4.38 (2H, m, N-CH_2), 6.57 (1H, d, J = 2.4 Hz, C-4-H), 6.63 (1H, dd, J = 8.7 Hz, J = 2.4 Hz, C-2-H), 7.11 (1H, d, J = 8.7 Hz, C-1-H) and 7.25 (1H, s, C-5′-H); MS *m/z* (FAB+) 462.1 [100, $(M+H)^+$], 73.0 [79]; Acc MS *m/z* (FAB+) 462.2922, $C_{28}H_{40}N_3OSi$ requires 462.2941.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(1′-propionitrile)-pyrazole (95)

A 1.0 M solution of TBAF in anhydrous THF (110 µL, 110 µmol) was added to a stirred solution of **93** (25 mg, 50 µmol) in anhydrous THF (5 mL) at room temperature, under an atmosphere of N_2. The mixture was stirred for 3 hours at room temperature, after which the solvent was removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (70 mL), washed with H_2O (20 mL), then brine (20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude yellow oil was purified by flash chromatography (DCM/EtOAc, 8:2) to give **95** as a light yellow oil (12 mg, 63%). This product was then triturated with EtOAc/hexane to give a light yellow powder (8 mg): mp 92-94°C; IR (KBr) 3230 (OH), 3095 (arom CH), 2925-2860 (aliph CH), 2265 (CN), 1665-1500 (C=N and arom C=C), 1245 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.94 (3H, s, C-18-H_3), 1.34-2.60 (11H, m), 2.73-2.94 (4H, m, C-6-H_2 and CH_2CN), 4.19-4.31

(2H, m, N-CH_2), 4.92 (1H, br s, exchanged with D_2O, OH), 6.50 (1H, d, J = 2.7 Hz, C-4-H), 6.55 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.02 (1H, s, C-5'-H) and 7.08 (1H, d, J = 8.4 Hz, C-1-H); MS *m/z* (FAB+) 348.1 [90, $(M+H)^+$], 147.1 [64], 73.0 [100]; Acc MS *m/z* (FAB+) 348.2072, $C_{22}H_{26}N_3O$ requires 348.2076. HPLC (MeOH/H_2O, 90:10, λ_{max} = 229.8 nm) Rt = 2.27 min, 96%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(2'-propionitrile)-pyrazole (96)

Method A: A 1.0 M solution of TBAF in anhydrous THF (150 µL, 150 µmol) was added to a stirred solution of **94** (35 mg, 76 µmol) in anhydrous THF (5 mL) at room temperature, under an atmosphere of N_2. The mixture was stirred for 3 hours at room temperature, after which the solvent was removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (70 mL), washed with H_2O (20 mL), then brine (20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude brown oil was purified by flash chromatography (DCM/EtOAc, 8:2) to give **96** as a light brown oil (10 mg, 38%).

Method B: A 1.0 M solution of TBAF in anhydrous THF (916 µL, 916 µmol) was added to a stirred solution of **99** (220 mg, 458 µmol) in anhydrous THF (20 mL) at room temperature, under an atmosphere of N_2. The mixture was stirred overnight at room temperature after which the solvent was removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc/$CHCl_3$, 9:1) to give **96** as a pale pink foam (136 mg, 85%). This compound was then triturated with EtOAc/Hexane to give white crystals (99 mg, 62%): mp 198-200°C; IR (KBr) 3160 (br, OH), 2925-2860 (aliph CH), 2250 (CN), 1610-1500 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.06 (3H, s, C-18-H_3), 1.41-2.63 (11H, m), 2.81-3.05 (4H, m, C-6-H_2 and CH_2CN), 4.22-4.34 (2H, m, N-CH_2), 5.42 (1H, br s, exchanged with D_2O, OH), 6.57 (1H, d, J = 2.4 Hz, C-4-H), 6.64 (1H, dd, J = 8.3 Hz, J = 2.4 Hz, C-2-H), 7.12 (1H, d, J = 8.3 Hz, C-1-H) and 7.25 (1H, s, C-5'-H); δ_C ($CDCl_3$, 100.4 MHz) 18.6 (q, C-18), 19.7 (t, CH_2CN), 24.5 (t), 26.5 (t), 27.6 (t), 29.8 (t), 34.8 (t), 37.7 (d), 42.4 (s, C-13), 44.4 (d), 45.9 (t, N-CH_2), 62.2 (d), 113.1 (d), 115.7 (d), 117.4 (s), 124.3 (s), 126.4 (d), 132.0 (s), 135.2 (d), 138.2 (s), 154.1 (s) and 157.98 (s); MS *m/z* (FAB+) 348.1 [100, $(M+H)^+$], 147.0

[50], 85.1 [75], 73.0 [94]; Acc MS *m/z* (FAB+) 348.2060, $C_{22}H_{26}N_3O$ requires 348.2076. Found: C, 75.90; H, 7.17; N, 12.10. $C_{22}H_{25}N_3O$ requires: C, 76.05; H, 7.25; N, 12.09%.

16-hydroxymethylene-estrone (97)

A 1.0 M solution of TBAF in anhydrous THF (21.3 mL, 21.3 mmol) was added to a stirred solution of **91** (4.4 g, 10.7 mmol) in anhydrous THF (100 mL) at room temperature, under an atmosphere of N_2. The mixture was stirred overnight to give a brown solution. After removal of the solvent under reduced pressure, H_2O (300 mL) was added and the organics were extracted with EtOAc (2×200 mL), washed with H_2O (200 mL), then brine (200 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The light yellow crude product was triturated with boiling EtOAc to give **97** as a pale yellow powder (2.21 g, 69%): mp 218-220°C [lit.[229] (acetone) 229-231°C]; IR (KBr) 3415, 3305 (br, OH), 2920-2870 (aliph CH), 1712 (C=O), 1630-1450 (C=C and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.80 (3H, s, C-18-H_3), 1.28-2.61 (11H, m), 2.69-2.78 (2H, m, C-6-H_2), 6.44 (1H, d, J = 2.6 Hz, C-4-H), 6.51 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.04 (1H, d, J = 8.5 Hz, C-1-H), 7.38 (1H, s, =C*H*OH), 9.02 (1H, s, exchanged with D_2O, C-3-OH) and 10.69 (1H, br s, exchanged with D_2O, =CHO*H*); MS *m/z* (FAB+) 299.2 [36, $(M+H)^+$], 242.3 [100, $(M+H\text{-}C(CH_3)_3)^+$]; Acc MS *m/z* (FAB+) 299.1650, $C_{19}H_{23}O_3$ requires 299.1647.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-pyrazole (98)

Hydrazine hydrate (48 µL, 1.00 mmol) was added to a solution of **97** (200 mg, 670 µmol) in EtOH (10 mL) at room temperature, under an atmosphere of N_2. The resulting intense yellow solution was heated to reflux for 30 minutes. After cooling down, the solvent was removed under reduced pressure, H_2O (50 mL) added and the mixture acidified with 5M HCl. The organics were extracted with EtOAc (150 mL), washed with H_2O (2×50 mL), then brine (2×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a pale yellow product (212 mg). The crude material was recrystallised from EtOH to yield **98** as pale yellow flaky crystals (125 mg, 64%): mp 326-328°C [lit.[219]320-328°C]; IR (KBr) 3300, 3125 (NH, OH), 2934-2855 (aliph CH), 1615-1500 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.90 (3H, s, C-18-H_3), 1.32-2.58 (11H, m), 2.69-2.84 (2H, m, C-6-H_2), 6.45

(1H, d, J = 2.6 Hz, C-4-H), 6.52 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H), 7.06 (1H, d, J = 8.4 Hz, C-1-H), 7.26 (1H, s, C-5′-H), 9.02 (1H, s, exchanged with D_2O, C-3-OH) and 12.00 (1H, d, J = 3.5 Hz, exchanged with D_2O, NH); MS *m/z* (FAB+) 295.0 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 295.1808, $C_{19}H_{23}N_2O$ requires 295.1810.

3-*O-tert*-Butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-c]-(2′-propionitrile-3′,4′-dihydro)-pyrazol-3′-ol (99)

Cyanoethylhydrazine (177 µL, 2.18 mmol) was added to a stirred solution of **91** (600 mg, 1.45 mmol) in EtOH (50 mL) under an atmosphere of N_2. After 4 hours of stirring at room temperature, the resulting orange mixture was concentrated under reduced pressure and H_2O (100 mL) added, followed by 5M HCl. The organics were extracted with EtOAc (2×100 mL), washed with H_2O (50 mL), then brine (50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 7:3) to give **99** as a pale yellow solid (510 mg, 73%). This compound was recrystallised from EtOH to give white needles (240 mg, 34%): mp 145-147°C; IR (KBr) 3380 (OH), 2950-2860 (aliph CH), 2260 (CN), 1610, 1495 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.15 (6H, s, $Si(CH_3)_2$), 0.88 (3H, s, C-18-H_3), 0.93 (9H, s, $C(CH_3)_3$), 1.19-3.16 (11H, m), 2.70-3.16 (7H, m), 5.97 (1H, s, exchanged with D_2O, OH), 6.48 (1H, d, J = 2.7 Hz, C-4-H), 6.56 (1H, dd, J = 8.2 Hz, J = 2.7 Hz, C-2-H), 6.74 (1H, d, J = 1.9 Hz, C-5′-H) and 7.10 (1H, d, J = 8.2 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) -4.4 (2×q, $Si(CH_3)_2$), 15.6 (q, C-18), 17.1 (t, CH_2CN), 18.0 (s, $C(CH_3)_3$), 25.6 (3×q, $C(CH_3)_3$), 25.8 (t), 27.0 (t), 29.1 (t), 29.4 (t), 31.4 (t), 38.6 (d), 43.2 (d), 43.9 (t, N-CH_2), 47.1 (s, C-13), 49.5 (d), 56.7 (d), 104.2 (s), 116.8 (d), 119.4 (d), 119.9 (s, CN), 126.0 (d), 132.5 (s), 137.3 (s), 144.6 (s) and 152.4 (s); MS *m/z* (FAB+) 480.1 [100, $(M+H)^+$], 462.1 [63, $(M+H-H_2O)^+$], 72.9 [77]; MS *m/z* (FAB-) 632.3 [32, $(M+NBA)^-$]; Acc MS *m/z* (FAB+) 480.3038 $C_{28}H_{42}N_3O_2Si$ requires 480.3046.

3-Benzyloxy-16-(1′-hydroxy-ethylidene)-estra-1,3,5(10)-triene-17-one (100)

^{t}BuOK (438 mg, 4.14 mmol) was added to a stirred solution of **3** (500 mg, 1.38 mmol) in anhydrous toluene (6 mL) and anhydrous DMSO (1.5 mL) at 0°C, under an atmosphere of N_2. Ethyl acetate (1.27 mL, 17.94 mmol) was then added and the mixture was heated to reflux for 1 hour. After cooling down, the resulting brown

solution was poured into H_2O (200 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×150 mL), washed with H_2O (2×100 mL), then brine (2×100 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (DCM) to yield **100** as a light yellow crystalline solid (447 mg, 80%). This compound was recrystallised from EtOH to give yellow crystals (351 mg, 63%): mp 126-128°C; IR (KBr) 2930-2860 (aliph CH), 1660 (C=O), 1615-1455 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.83, 0.95 (3H, 2×s, C-18-H_3), 1.38-2.49 (11H, m), 1.97, 2.37 (3H, 2×s, C-2'-H_3), 2.87-2.92 (2H, m, C-6-H_2), 3.43 (app dd, J = 9.4 Hz, J = 8.2 Hz, C-16-H, ketone tautomer), 5.02 (2H, s, OCH_2Ar), 6.71 (1H, d, J = 2.8 Hz, C-4-H), 6.77 (1H, dd, J = 8.3 Hz, J = 2.8 Hz, C-2-H), 7.17 (1H, d, J = 8.3 Hz, C-1-H), 7.28-7.42 (5H, m, C_6H_5) and 12.91 (br s, exchanged with D_2O, C-1'-OH, enol tautomer); MS *m/z* (FAB+) 403.1 [68, $(M+H)^+$], 91.0 [100, $(CH_2Ar)^+$]; Acc MS *m/z* (FAB+) 403.2246, $C_{27}H_{31}O_3$ requires 403.2273. Found: C, 80.20; H, 7.56. $C_{27}H_{30}O_3$ requires: C, 80.56; H, 7.51%.

3-*O-tert*-Butyl-dimethylsilyl-16-(1'-hydroxy-2',2',2'-trifluoro-ethylidene)-estrone (101)

^{t}BuOK (875 mg, 7.80 mmol) was added to a stirred solution of **90** (1.0 g, 2.60 mmol) in anhydrous toluene (20 mL) at 0°C, under an atmosphere of N_2. After 20 minutes of stirring, ethyl trifluoroacetate (2.16 mL, 18.2 mmol) was added and the mixture heated to reflux for 2 hours. After cooling down, the resulting dark orange suspension was poured into H_2O (50 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude orange oil that crystallised on standing was used without further purification (1.4 g, quant.). An analytical sample was recrystallised from EtOH to give **101** as light yellow crystals: mp 145-146°C; IR (KBr) 2930-2860 (aliph CH), 1690 (C=O), 1640-1495 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.19 (6H, s, $Si(CH_3)_2$), 0.98 (9H, s, $C(CH_3)_3$), 0.99 (3H, s, C-18-H_3), 1.42-2.90 (13H, m), 6.57 (1H, d, J = 2.6 Hz, C-4-H), 6.63 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H) and 7.11 (1H, d, J = 8.4 Hz, C-1-H), C-1'-OH not seen; δ_C ($CDCl_3$, 100.4 MHz) -4.4 (2×q, $Si(CH_3)_2$), 14.8 (q, C-18), 18.1 (s, $C(CH_3)_3$), 25.2 (t), 25.6 (t), 25.7 (3×q, $C(CH_3)_3$), 26.7 (t), 29.3 (t), 31.0 (t), 37.5 (d), 43.9 (d), 48.5 (s, C-13), 49.5 (d), 110.9 (s, C-16), 117.4 (d), ~ 119

(app d, J = 272 Hz, CF_3), 120.0 (d), 126.0 (d), 132.0 (s), 137.4 (s), 153.6 (s, C-3), ~ 154 (app d, J = 38 Hz, C-1′) and 216.0 (s, C=O); MS *m/z* (FAB+) 480.1 [47, M^+], 423.0 [38, $(M\text{-}F_3)^+$], 73.0 [100]; Acc MS *m/z* (FAB+) 480.2296, $C_{26}H_{35}F_3O_3Si$ requires 480.2308. Found: C, 64.90; H, 7.13. $C_{26}H_{35}F_3O_3Si$ requires: C, 64.97; H, 7.34%.

3-*O-tert*-Butyl-dimethylsilyl-16-(1′-hydroxy-1″-pyridin-3″-ylmethylene)-estrone (102)

^{t}BuOK (875 mg, 7.80 mmol) was added to a stirred solution of **90** (1.0 g, 2.60 mmol) in anhydrous toluene (30 mL) at 0°C under an atmosphere of N_2. After 20 minutes of stirring, ethyl nicotinate (2.48 mL, 18.2 mmol) was added and the mixture was heated to reflux for 1.5 hours. After cooling down, the resulting dark red suspension was poured into H_2O (200 mL) and acidified with 5M HCl. The organics were extracted with EtOAc (2×100 mL), washed with H_2O (100 mL), then brine (2×100 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude yellow solid was recrystallised from EtOH to give **102** as white flaky crystals (985 mg, 77%): mp 169-170°C; IR (KBr) 2930-2855 (aliph CH), 1660 (C=O), 1605, 1495 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.21 (6H, s, $Si(CH_3)_2$), 0.99 (9H, s, $C(CH_3)_3$), 1.11 (3H, s, C-18-H_3), 1.38-2.77 (11H, m), 2.81-2.95 (2H, m, C-6-H_2), 6.58 (1H, d, J = 2.6 Hz, C-4-H), 6.63 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.12 (1H, d, J = 8.5 Hz, C-1-H), 7.41 (1H, ddd, J = 8.1 Hz, J = 4.9 Hz, J = 0.8 Hz, C-5″-H), 8.09 (1H, app dt, J = 8.1 Hz, J = 2.0 Hz, C-4″-H), 8.69 (1H, dd, J = 4.9 Hz, J = 1.9 Hz, C-6″-H), 8.97 (1H, app dd, J = 2.1 Hz, J = 0.8 Hz, C-2″-H) and 13.63 (1H, s, exchanged with D_2O, C-1′-OH); MS *m/z* (FAB+) 490.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 490.2775, $C_{30}H_{40}NO_3Si$ requires 490.2777.

16-(1′-hydroxy-1″-pyridin-3″-ylmethylene)-estrone (103)

A 1.0 M solution of TBAF in anhydrous THF (1.43 mL, 1.43 mmol) was added to a stirred solution of **102** (350 mg, 716 μmol) in anhydrous THF (10 mL) under an atmosphere of N_2. The resulting light brown solution was stirred at room temperature for 3 hours, after which the solvent was removed under reduced pressure and H_2O added (100 mL), followed by AcOH glacial (5 drops). The organics were extracted with EtOAc (100 mL), Et_2O (100 mL) and $CHCl_3$ (100 mL), washed with H_2O (100

mL), then brine (100 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was triturated with boiling EtOH to give **103** as a pale yellow solid (125 mg, 50%): mp 275-278°C; IR (KBr) 2950-2855 (aliph CH), 1645 (C=O), 1610-1500 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.09 (3H, s, C-18-H_3), 1.39-2.76 (11H, m), 2.85-2.91 (2H, m, C-6-H_2), 5.48 (1H, s, exchanged with D_2O, C-3-OH), 6.59 (1H, d, *J* = 2.7 Hz, C-4-H), 6.65 (1H, dd, *J* = 8.4 Hz, *J* = 2.7 Hz, C-2-H), 7.15 (1H, d, *J* = 8.4 Hz, C-1-H), 7.41 (1H, ddd, *J* = 8.1 Hz, *J* = 4.9 Hz, *J* = 0.8 Hz, C-5″-H), 8.09 (1H, dt, *J* = 8.1 Hz, *J* = 1.8 Hz, C-4″-H), 8.67 (1H, dd, *J* = 4.9 Hz, *J* = 1.8 Hz, C-6″-H), 8.94 (1H, app d, *J* = 1.8 Hz, C-2″-H) and 13.58 (1H, s, exchanged with D_2O, C-1′-OH); MS *m/z* (FAB+) 376.0 [100, $(M+H)^+$], 242.1 [54]; Acc MS *m/z* (FAB+) 376.1903, $C_{24}H_{26}NO_3$ requires 376.1913. Found: C, 74.70; H, 6.80; N, 3.51. $C_{24}H_{25}NO_3.(H_2O)_{1/2}$ requires: C, 74.98; H, 6.82; N, 3.64%.

16-(1′-hydroxy-ethylidene)-estrone (104)

Following method 2, a suspension of **100** (200 mg, 497 μmol) and Pd-C (10%, 80 mg) in MeOH/THF 2:1 (30 mL) was hydrogenated for 2 hours to give **104** as a white solid (98 mg, 63%). This product was recrystallised from acetone/hexane to give white crystals (72 mg, 46%): mp 211-214°C; IR (KBr) 3450 (OH), 2915-2860 (aliph CH), 1705 (C=O), 1655-1510 (C=O, C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.83, 0.96 (3H, 2×s, C-18-H_3), 1.39-2.40 (11H, m), 1.98, 2.37 (3H, 2×s, C-2′-H_3), 2.83-2.89 (2H, m, C-6-H_2), 3.43 (dd, *J* = 9.4 Hz, *J* = 8.2 Hz, C-16-H, ketone tautomer), 4.70 (1H, br s, exchanged with D_2O, C-3-OH), 6.55-6.57 (1H, m, C-4-H), 6.62 (1H, dd, *J* = 8.0 Hz, *J* = 2.7 Hz, C-2-H) and 7.11 (1H, d, *J* = 8.0 Hz, C-1-H), C-1′-OH (enol tautomer) not seen; MS *m/z* (FAB+) 313.1 [100, $(M+H)^+$], 73.0 [19]; Acc MS *m/z* (FAB+) 313.1799, $C_{20}H_{25}O_3$ requires 313.1804. Found: C, 76.60; H, 7.82. $C_{20}H_{24}O_3$ requires: C, 76.89; H, 7.74%.

16-(1′-hydroxy-2′,2′,2′-trifluoro-ethylidene)-estrone (105)

A 1.0 M solution of TBAF in anhydrous THF (4.90 mL, 4.90 mmol) was added to a stirred solution of **101** (1.18 g, 2.45 mmol, crude product) in anhydrous THF (50 mL) under an atmosphere of N_2. The resulting light brown solution was stirred at room temperature for 4 hours, after which the solvent was removed under reduced pressure and H_2O added (100 mL). The organics were extracted with EtOAc (2×100

mL), washed with H_2O (2×80 mL), then brine (2×80 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (DCM) to give **105** as a light brown oil (535 mg, 60%). This compound was crystallised from cold DCM to give white crystals (249 mg, 28%): mp 91-93°C; IR (KBr) 3430 (OH), 2930-2860 (aliph CH), 1685 (C=O), 1610, 1500 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.02 (3H, s, C-18-H_3), 1.43-2.91 (13H, m), 6.58 (1H, d, J = 2.7 Hz, C-4-H), 6.64 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H) and 7.14 (1H, d, J = 8.4 Hz, C-1-H), C-3-OH and C-1′-OH not seen; MS *m/z* (FAB+) 366.1 [100, M^+]; Acc MS *m/z* (FAB+) 366.1476, $C_{20}H_{21}F_3O_3$ requires 366.1443. HPLC (MeOH/H_2O, 96:4, λ_{max} = 280.5 nm) Rt = 1.66 min, 100%. Found: C, 57.60; H, 5.37. $C_{20}H_{21}F_3O_3.(CH_2Cl_2)_{3/4}$ requires: C, 57.95; H, 5.27%.

3-*O*-*tert*-Butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-c]-[5′-(1″-pyridin-3″-yl)-3′,4′-dihydro)]-pyrazol-3′-ol (106)

Hydrazine hydrate (75 μL, 1.53 mmol) was added to a refluxing solution of **102** (500 mg, 1.02 mmol) in EtOH (20 mL), under an atmosphere of N_2. The resulting pale yellow solution was heated to reflux for 1 hour, after which the solvent was partially removed under reduced pressure and H_2O (100 mL) added. The resulting white precipitate was filtered and dried (460 mg, 89%) and an analytical sample was recrystallised from EtOH/H_2O to give **106** as white crystals: mp 144-145°C; IR (KBr) 3480, 3330, 3190 (NH and OH), 2940-2860 (aliph CH), 1610-1495 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.14 (6H, s, $Si(CH_3)_2$), 0.92 (9H, s, $C(CH_3)_3$), 0.94 (3H, s, C-18-H_3), 1.12-2.31 (11H, m), 2.63-2.71 (2H, m, C-6-H_2), 3.43 (1H, app d, J = 9.0 Hz, C-16-H), 5.97 (1H, s, exchanged with D_2O, C-17-OH), 6.46 (1H, d, J = 2.5 Hz, C-4-H), 6.55 (1H, dd, J = 8.4 Hz, J = 2.5 Hz, C-2-H), 7.10 (1H, d, J = 8.4 Hz, C-1-H), 7.26 (1H, s, exchanged with D_2O, NH), 7.35 (1H, app ddd, J = 8.1 Hz, J = 4.6 Hz, J = 0.8 Hz, C-5″-H), 7.91 (1H, app dt, J = 8.1 Hz, J = 1.7 Hz, C-4″-H), 8.43 (1H, dd, J = 4.6 Hz, J = 1.7 Hz, C-6″-H) and 8.74 (1H, app d, J = 1.7 Hz, C-2″-H); MS *m/z* (FAB+) 504.1 [100, $(M+H)^+$], 486.1 [27, $(M+H-H_2O)^+$]; Acc MS *m/z* (FAB+) 504.3045, $C_{30}H_{42}N_3O_2Si$ requires 504.3046.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(5′-methyl)-pyrazole (107)

Hydrazine hydrate (56 μL, 1.15 mmol) was added to a refluxing solution of **104** (240 mg, 768 μmol) in EtOH (15 mL) under an atmosphere of N_2. The resulting pale yellow solution was heated to reflux for 45 minutes, after which the solvent was removed under reduced pressure, H_2O (50 mL) added and the mixture acidified with 5M HCl. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (DCM/EtOAc, 8:2) to give **107** as a pale brown solid (84 mg, 35%). This was triturated with acetone to give an off-white solid (32 mg, 13%): mp 234-236°C; IR (KBr) 3295 (OH), 2930-2860 (aliph CH), 1620-1500 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.34-2.64 (11H, m), 2.71-2.85 (2H, m, C-6-H_2), 2.28 (3H, s, C-1″-H_3), 6.45 (1H, d, J = 2.6 Hz, C-4-H), 6.52 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.04 (1H, d, J = 8.6 Hz, C-1-H) and 9.04 (1H, s, exchanged with D_2O, NH or OH), NH or OH not seen; MS *m/z* (FAB+) 309.2 [100, $(M+H)^+$], 95.1 [33], 69.0 [39]; Acc MS *m/z* (FAB+) 309.1978, $C_{20}H_{25}N_2O$ requires 309.1967. HPLC (MeOH/H_2O, 96:4, λ_{max} = 280.5 nm) Rt = 1.98 min, 99%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-(5′-trifluoro-methyl)-pyrazole (108)

Hydrazine hydrate (32 μL, 655 μmol) was added to a refluxing solution of **105** (160 mg, 437 μmol) in EtOH (15 mL) under an atmosphere of N_2. The resulting pale yellow solution was heated to reflux for 3 hours, after which *p*-toluene sulfonic acid (~ 10 mg) was added. The mixture was then heated to reflux overnight and after cooling down, the solvent was removed under reduced pressure, H_2O (50 mL) added, followed by 5M HCl. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (DCM/EtOAc, 8:2) to give **108** as a pale yellow solid (95 mg, 60%). This compound was precipitated from DCM/Hexane (1:5) to give an off-white solid (51 mg, 32%): mp 152-155°C; IR (KBr) 3220 (br, OH), 2930-2860 (aliph CH), 1610-1500 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.40-2.74 (11H, m), 2.78-2.89 (2H, m, C-6-H_2), 4.63 (1H, s, exchanged with D_2O, C-3-OH), 6.53

(1H, d, J = 2.7 Hz, C-4-H), 6.59 (1H, dd, J = 8.2 Hz, J = 2.7 Hz, C-2-H) and 7.08 (1H, d, J = 8.2 Hz, C-1-H), NH not seen; MS *m/z* (FAB+) 363.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 363.1689, $C_{20}H_{22}N_2F_3O$ requires 363.1684. HPLC ($MeOH/H_2O$, 85:15, λ_{max} = 280.5 nm) Rt = 2.43 min, 98%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-c]-[5′-(1″-pyridin-3″-yl)]-pyrazole (109)

A 1.0 M solution of TBAF in anhydrous THF (436 µL, 436 µmol) was added to a stirred solution of **106** (110 mg, 218 µmol) in anhydrous THF (10 mL) at room temperature under an atmosphere of N_2. The resulting light brown solution was stirred overnight at room temperature, after which the solvent was removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude foam was triturated with boiling EtOAc/$CHCl_3$ (1:1) to give **109** as a white powder (61 mg, 75%): mp 298-300°C (dec.); IR (KBr) 2980-2840 (aliph CH), 1610-1495 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.98 (3H, s, C-18-H_3), 1.37-2.87 (13H, m), 6.45 (1H, d, J = 2.6 Hz, C-4-H), 6.51 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.05 (1H, d, J = 8.5 Hz, C-1-H), 7.37-7.48 (1H, m, C-4″-H or C-5″-H), 7.97-8.06 (1H, m, C-4″-H or C-5″-H), 8.42-8.49 (1H, m, C-2″-H or C-6″-H) and 8.84-8.89 (1H, m, C-2″-H or C-6″-H), 9.01 (1H, s, exchanged with D_2O, OH), 12.65 and 12.72 (1H, 2×s, exchanged with D_2O, NH); MS *m/z* (FAB+) 372.0 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 372.2080, $C_{24}H_{26}N_3O$ requires 372.2076.

5.4.6 Other D-ring fused derivatives of E1

3-Hydroxy-17-*O*-allyl-oximino-estra-1,3,5(10)-triene (110)

NaOAc (3 g, 10.17 mmol) followed by *O*-allyl-hydroxylamine hydrochloride (4.5 g, 41.44 mmol) were added to a solution of E1 (1.0 g, 3.70 mmol) in a mixture of $MeOH/H_2O$ (5:1, 180 mL). The resulting solution was stirred at room temperature overnight, after which the solvent was removed under reduced pressure and H_2O added (200 mL). The organics were extracted with EtOAc (300 mL), washed with H_2O (2×100 mL), then brine (2×100 mL), dried (Na_2SO_4), filtered and concentrated

under reduced pressure to give a white product (1.37 g). The crude material was recrystallised from MeOH/H_2O to give **110** as white crystals (1.13 g, 94%): mp 81-83°C; IR (KBr) 3455 (OH), 3185 (arom CH), 2955-2845 (aliph CH), 1640-1505 (C=N, C=C and arom C=C), 1240 cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.94 (3H, s, C-18-H_3), 1.35-2.60 (13H, m), 2.80-2.87 (2H, m, C-6-H_2), 4.50-4.60 (2H, m, C-1′-H_2), 4.74 (1H, s, exchanged with D_2O, OH), 5.19 (1H, app dq, J_{cis} = 10.3 Hz, J ~ 1.5 Hz, C-3′-H), 5.28 (1H, app dq, J_{trans} = 17.2 Hz, J ~ 1.5 Hz, C-3′-H), 5.59-6.01 (1H, m, C-2′-H), 6.56 (1H, d, J = 2.7 Hz, C-4-H), 6.63 (1H, dd, J = 8.2 Hz, J = 2.7 Hz, C-2-H) and 7.15 (1H, d, J = 8.2 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 17.7 (q, C-18), 23.4 (t), 26.5 (t), 26.6 (t), 27.6 (t), 30.0 (t), 34.5 (t), 38.5 (d), 44.3 (d), 44.8 (s, C-13), 53.3 (d), 74.6 (t, C-1′), 113.0 (d), 115.5 (d), 117.3 (t, C-3′), 126.7 (d), 132.4 (s), 134.7 (d, C-2′), 138.2 (s), 153.7 (s, C-3) and 171.3 (s, C=N); MS m/z (FAB+) 326.1 [100, $(M+H)^+$], 268.1 [30, $OCH_2CH{=}CH_2^+$]; Acc MS m/z (FAB+) 326.2116, $C_{21}H_{28}NO_2$ requires 326.2120. HPLC (MeOH/H_2O, 90:10; λ_{max} = 279.3 nm) Rt = 3.01 min, 99%. Found: C, 76.40; H, 8.24; N, 4.36. $C_{21}H_{27}NO_2$ requires: C, 76.23; H, 8.50; N, 4.17%.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-b]-pyridine (111)

17-*O*-Allyl-oxime **110** (2.45 g, 7.53 mmol) was stirred and heated to 230°C (using a sand bath) for 46 hours. The resulting dark brown solid was allowed to cool down and EtOH was added until most of the solid was dissolved. The remaining undissolved material was crushed to a fine powder, silica was added and the solvent removed under reduced pressure. This was purified by flash chromatography ($CHCl_3$/EtOAc, 9:1) to give **111** as a dark orange solid (200 mg, 9%). A second flash chromatography ($CHCl_3$/EtOAc, 95:5) yielded a light orange powder (146 mg, 6%). For analysis, a sample was triturated with $CHCl_3$ to give an off-white powder: mp 279-284°C (dec.); IR (KBr) 3415 (OH), 3115-3025 (arom CH), 2985-2855 (aliph CH), 1615-1500 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.36-2.83 (13H, m), 6.47 (1H, d, J = 2.4 Hz, C-4-H), 6.54 (1H, dd, J = 8.4 Hz, J = 2.4 Hz, C-2-H), 7.05-7.09 (2H, m, C-1-H and C-5′-H), 7.60 (1H, d, J = 7.4 Hz, C-4′-H), 8.25 (1H, app d, J = 4.3 Hz, C-6′-H) and 9.05 (1H, s, exchanged with D_2O, OH); δ_H ($CDCl_3$, 400 MHz) 1.01 (3H, s, C-18-H_3), 1.43-2.96 (13H, m), 6.08 (1H, br s, OH), 6.61 (1H, d, J = 2.6 Hz, C-4-H), 6.67 (1H, dd, J = 8.4 Hz, J = 2.6 Hz,

C-2-H), 7.04 (1H, dd, *J* = 7.4 Hz, *J* = 4.8 Hz, C-5′-H), 7.18 (1H, d, *J* = 8.4 Hz, C-1-H), 7.54 (1H, d, *J* = 7.4 Hz, C-4′-H) and 8.33 (1H, d, *J* = 4.8 Hz, C-6′-H); δ_C (DMSO-d_6, 100.4 MHz) 18.5 (q, C-18), 26.9 (t), 27.9 (t), 29.9 (t), 30.4 (t), 34.4 (t), 38.2 (d), 44.7 (d), 46.3 (s, C-13), 55.4 (d), 113.5 (d), 115.7 (d), 121.7 (d), 126.5 (d), 130.9 (s), 133.2 (d), 136.4 (s), 137.7 (s), 147.2 (d), 155.6 (s, C-3) and 173.1 (s, C=N); MS *m/z* (FAB+) 306.1 [85, $(M+H)^+$], 207.0 [95], 114.9 [100]; Acc MS *m/z* (FAB+) 306.1864, $C_{21}H_{24}NO$ requires 306.1858. HPLC (MeOH/H_2O, 80:20; λ_{max} = 268.7 nm) Rt = 2.90 min, 98%. Found: C, 77.00; H, 7.16; N, 4.12. $C_{21}H_{23}NO.(CHCl_3)_{1/5}$ requires: C, 77.33; H, 7.10; N, 4.25%.

3-*O*-*tert*-Butyl-dimethylsilyl-16-ethoxymethylene-estrone (112) and 3-*O*-*tert*-butyl-dimethylsilyl-estra-1,3,5(10)-triene-[17,16-f]-(tetrazolo-[1,5-a]-pyrimidine) (113)

5-Aminotetrazole (187 mg, 1.82 mmol) was added to a stirred suspension of **91** (500 mg, 1.21 mmol) in EtOH (50 mL) and the resulting mixture heated to reflux for 48 hours. After cooling down, H_2O was added (50 mL) and the organics were extracted with EtOAc (2×50 mL), washed with H_2O (30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude mixture was purified by flash chromatography ($CHCl_3$/EtOAc, 95:5) to give two products:

The less polar fraction gave **112** as an off-white oil that crystallised on standing (231 mg, 43%): mp 144-146°C; IR (KBr) 2955-2855 (aliph CH), 1710 (C=O), 1635-1495 (C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.21 (6H, s, $Si(CH_3)_2$), 0.94 (3H, s, C-18-H_3), 0.99 (9H, s, $C(CH_3)_3$), 1.36 (3H, t, *J* = 7.0 Hz, C-3′-H_3), 1.39-2.96 (13H, m), 4.04-4.85 (2H, m, C-2′-H_2), 6.56 (1H, d, *J* = 2.7 Hz, C-4-H), 6.62 (1H, dd, *J* = 8.4 Hz, *J* = 2.7 Hz, C-2-H), 7.11 (1H, d, *J* = 8.4 Hz, C-1-H) and 7.28-7.30 (1H, m, C-1′-H); MS *m/z* (FAB+) 441.6 [100, $(M+H)^+$], 73.1 [38]; Acc MS *m/z* (FAB+) 441.2805, $C_{27}H_{41}O_3Si$ requires 441.2825.

The more polar fraction gave **113** as a white solid (179 mg, 32%). An analytical sample was recrystallised from EtOH to give white crystals: mp 203-205°C; IR (KBr) 2930-2855 (aliph CH), 1640-1495 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.22 (6H, s, $Si(CH_3)_2$), 1.00 (9H, s, $C(CH_3)_3$), 1.18 (3H, s, C-18-H_3), 1.52-3.13

(13H, m), 6.60 (1H, d, J = 2.7 Hz, C-4-H), 6.66 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.16 (1H, d, J = 8.4 Hz, C-1-H) and 8.74-8.75 (1H, m, C-7′-H); MS *m/z* (FAB+) 462.5 [100, $(M+H)^+$], 73.0 [48]; Acc MS *m/z* (FAB+) 462.2687, $C_{26}H_{36}N_5OSi$ requires 462.2689.

3-Hydroxy-estra-1,3,5(10)-triene-[17,16-f]-(tetrazolo-[1,5-a]-pyrimidine) (114)

A 1.0 M solution of TBAF in anhydrous THF (650 µL, 650 µmol) was added to a stirred solution of **113** (150 mg, 325 µmol) in anhydrous THF (15 mL) at room temperature, under an atmosphere of N_2. The resulting light brown solution was stirred overnight at room temperature, after which H_2O was added (50 mL). The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×50 mL), then brine (2×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was triturated with boiling EtOAc to give **114** as an orange powder (80 mg, 71%): mp > 340°C; IR (KBr) 3290 (OH), 2930-2860 (aliph CH), 1642-1505 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.08 (3H, s, C-18-H_3), 1.22-3.04 (13H, m), 6.46 (1H, d, J = 2.4 Hz, C-4-H), 6.53 (1H, dd, J = 8.5 Hz, J = 2.4 Hz, C-2-H), 7.08 (1H, d, J = 8.5 Hz, C-1-H), 9.05 (1H, s, C-7′-H or OH) and 9.46 (1H, s, C-7′-H or OH); MS *m/z* (FAB+) 391.2 [71], 348.1 [100, $(M+H)^+$], 327.2 [80], 275.1 [50], 219.1 [84], 127.1 [55], 73.0 [69]; Acc MS *m/z* (FAB+) 348.1804, $C_{20}H_{22}N_5O$ requires 348.1824.

3-*O-tert*-Butyl-dimethylsilyl-estra-1,3,5(10),16-tetraene-[16,17-g]-(pyrazolo-[1,5-a]-pyrimidine) (115)

3-Aminopyrazole (60 mg, 727 µmol) was added to a stirred suspension of **91** (200 mg, 485 µmol) in EtOH (20 mL) and the resulting mixture heated to reflux for 15 hours. After cooling down, the final white suspension was filtered, washed with EtOH and air dried to give **115** as a white solid (171 mg, 77%): mp 239-241°C; IR (KBr) 2950-2855 (aliph CH), 1610-1405 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.22 (6H, s, $Si(CH_3)_2$), 1.00 (9H, s, $C(CH_3)_3$), 1.25 (3H, s, C-18-H_3), 1.53-3.04 (13H, m), 6.60 (1H, d, J = 2.7 Hz, C-4-H), 6.65 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 6.70 (1H, d, J = 2.3 Hz, C-3′-H), 7.17 (1H, d, J = 8.6 Hz, C-1-H), 8.13 (1H, d, J = 2.3 Hz, C-2′-H) and 8.43 (1H, s, C-5′-H); MS *m/z* (FAB+) 459.5 [100, M^+], 402.4

[38, $(M\text{-}C(CH_3)_3)^+$]; Acc MS *m/z* (FAB+) 459.2694, $C_{28}H_{37}N_3OSi$ requires 459.2706.

3-Hydroxy-estra-1,3,5(10),16-tetraene-[16,17-g]-(pyrazolo-[1,5-a]-pyrimidine) (116)

A 1.0 M solution of TBAF in anhydrous THF (566 µL, 566 µmol) was added to a stirred solution of **115** (130 mg, 283 µmol) in anhydrous THF (15 mL) at room temperature, under an atmosphere of N_2. The resulting light brown solution was stirred overnight at room temperature, after which H_2O was added (50 mL). The resulting white precipitate was filtered and air dried to give **116** as a white powder (98 mg, 100%). This product was recrystallised from EtOH/H_2O to give a white solid (89 mg, 91%): mp 333-335°C; IR (KBr) 3165 (br, OH), 2940-2860 (aliph CH), 1615-1515 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.14 (3H, s, C-18-H_3), 1.42-2.99 (13H, m), 6.46 (1H, d, *J* = 2.3 Hz, C-4-H), 6.52 (1H, dd, *J* = 8.2 Hz, *J* = 2.3 Hz, C-2-H), 6.69 (1H, d, *J* = 2.3 Hz, C-3′-H), 7.07 (1H, d, *J* = 8.2 Hz, C-1-H), 8.18 (1H, d, *J* = 2.3 Hz, C-2′-H), 8.50 (1H, s, C-5′-H) and 9.04 (1H, s, exchanged with D_2O, C-3-OH); MS *m/z* (FAB+) 346.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 346.1898, $C_{22}H_{24}N_3O$ requires 346.1919.

3-*O-tert*-Butyl-dimethylsilyl-estra-1,3,5(10),16-tetraene-[16,17-g]-(s-triazolo-[1,5-a]-pyrimidine) (117)

3-Amino-1,2,4-triazole (61 mg, 727 µmol) was added to a stirred suspension of **91** (200 mg, 485 µmol) in EtOH (20 mL) and the resulting mixture heated to reflux for 48 hours. After cooling down, H_2O was added (50 mL) and the organics were extracted with EtOAc (2×50 mL), washed with H_2O (30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The yellow crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 1:1) to give **117** as a white solid (111 mg, 50%): mp 162-164°C; IR (KBr) 2930-2855 (aliph CH), 1695-1495 (C=N and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.22 (6H, s, $Si(CH_3)_2$), 1.00 (9H, s, $C(CH_3)_3$), 1.26 (3H, s, C-18-H_3), 1.53-3.13 (13H, m), 6.60 (1H, d, *J* = 2.4 Hz, C-4-H), 6.66 (1H, dd, *J* = 8.3 Hz, *J* = 2.4 Hz, C-2-H), 7.16 (1H, d, *J* = 8.3 Hz, C-1-H), 8.50 (1H, s, C-2′-H or C-5′-H) and 8.72 (1H, s, C-2′-H or C-5′-H); δ_C ($CDCl_3$,

100.4 MHz) -4.2 (2×q, $Si(CH_3)_2$), 15.2 (q, C-18), 18.3 (s, $C(CH_3)_3$), 25.8 (3×q, $C(CH_3)_3$), 25.9 (t), 27.5 (t), 28.5 (t), 29.4 (t), 33.3 (t), 37.0 (d), 44.1 (d), 47.4 (s, C-13), 55.9 (d), 117.3 (d), 120.0 (d), 123.4 (s), 125.8 (d), 131.9 (s), 137.1 (s), 151.5 (d, C-2′ or C-5′), 153.4 (s), 155.6 (s), 156.1 (d, C-2′ or C-5′) and 159.0 (s); MS *m/z* (FAB+) 461.6 [100, $(M+H)^+$], 73.0 [20]; Acc MS *m/z* (FAB+) 461.2739, $C_{27}H_{37}N_4OSi$ requires 461.2737.

3-Hydroxy-estra-1,3,5(10),16-tetraene-[16,17-g]-(s-triazolo-[1,5-a]-pyrimidine) (118)

A 1.0 M solution of TBAF in anhydrous THF (738 µL, 738 µmol) was added to a stirred solution of **117** (170 mg, 369 µmol) in anhydrous THF (15 mL) at room temperature, under an atmosphere of N_2. The resulting light brown solution was stirred for 5.5 hours at room temperature, after which H_2O was added (50 mL). The resulting white precipitate was filtered and air dried to give **118** as a white powder (85 mg, 66%): mp > 340°C; IR (KBr) 3270 (br, OH), 2955-2860 (aliph CH), 1610-1505 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.15 (3H, s, C-18-H_3), 1.41-3.07 (13H, m), 6.46 (1H, d, J = 2.6 Hz, C-4-H), 6.53 (1H, dd, J = 8.2 Hz, J = 2.6 Hz, C-2-H), 7.07 (1H, d, J = 8.2 Hz, C-1-H), 8.66 (1H, s, C-2′-H or C-5′-H), 8.81 (1H, s, C-3′-H or C-5′-H) and 9.04 (1H, s, exchanged with D_2O, C-3-OH); MS *m/z* (FAB+) 347.2 [100, $(M+H)^+$], 242.3 [77], 219.2 [30], 73.0 [28]; Acc MS *m/z* (FAB+) 347.1859, $C_{21}H_{23}N_4O$ requires 347.1872.

5.4.7 Modifications at C6, C16 and C17 on E1 nucleus

3-Acetoxy-6-oxo-estra-1,3,5(10)-triene-17-one (119)

A solution of CrO_3 (673 mg, 6.73 mmol) in 10% AcOH (4 mL) was added dropwise over 2 hours to a stirred solution of **61** (500 mg, 1.60 mmol) in AcOH glacial, at 10-15°C in an ice/water bath. The resulting dark brown solution was stirred overnight at room temperature, after which the solvent was removed under reduced pressure and H_2O (100 mL) added. The organics were extracted with EtOAc (100 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a green foam. The crude product was

recrystallised from IPA to give **119** as grey crystals (103 mg, 20%): mp 191-194°C [lit.[252] (acetone) 193.5-195.5°C]; IR (KBr) 2950-2850 (aliph CH), 1765 (C=O), 1740 (C=O), 1675 (C=O), 1600, 1480 (arom C=C), 1200 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.20-2.63 (12H, m), 2.32 (3H, s, $COCH_3$), 2.88 (1H, dd, J_{AB} = 16.8 Hz, J = 3.1 Hz, C-7-H), 7.28 (1H, dd, J = 8.6 Hz, J = 2.6 Hz, C-2-H), 7.46 (1H, d, J= 8.6 Hz, C-1-H) and 7.77 (1H, d, J = 2.6 Hz, C-4-H); MS *m/z* (FAB+) 327.2 [100, $(M+H)^+$], 145.1 [27], 85.1 [50]; Acc MS *m/z* (FAB+) 327.1595, $C_{20}H_{23}O_4$ requires 327.1596.

6-Oxo-estrone (120)

A solution of KOH (247 mg, 4.40 mmol) in MeOH (1.5 mL) was added dropwise to a stirred solution of **119** (80 mg, 245 μmol) in MeOH (1.5 mL). The resulting brown solution was stirred for 2 hours at room temperature, after which the solvent was concentrated under reduced pressure and H_2O (20 mL) added, followed by 5M HCl. The organics were extracted with EtOAc (50 mL), washed with H_2O (20 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product (74 mg) was purified by flash chromatography ($CHCl_3$/EtOAc, 7:3) to give **120** as a light pink powder (41 mg, 58%). An analytical sample was triturated with boiling acetone to give an off-white solid: mp 241-243°C [lit.[269] (MeOH) 246-247°C]; IR (KBr) 3345 (br, OH), 2940-2880 (aliph CH), 1720 (C=O), 1680 (C=O), 1610-1495 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.93 (3H, s, C-18-H_3), 1.24-2.59 (12H, m), 2.87 (1H, dd, J_{AB} = 17.0 Hz, J = 3.3 Hz, C-7-H), 5.74 (1H, s, exchanged with D_2O, OH), 7.10 (1H, dd, J = 8.5 Hz, J = 2.8 Hz, C-2-H), 7.33 (1H, d, J = 8.5 Hz, C-1-H) and 7.60 (1H, d, J = 2.8 Hz, C-4-H); MS *m/z* (FAB+) 369.2 [50], 285.1 [95, $(M+H)^+$], 113.1 [68], 84.0 [94]; Acc MS *m/z* (FAB+) 285.1492, $C_{18}H_{21}O_3$ requires 285.1491. HPLC (MeOH/H_2O, 90:10, λ_{max} = 223.9 nm) Rt = 2.34 min, 100%.

3-Acetoxy-16-acetoxymethylene-estra-1,3,5(10)-triene-17-one (121)

Acetic anhydride (8.34 mL, 88.24 mmol) was added dropwise over 10 minutes to a stirred solution of **97** (230 mg, 771 μmol) in anhydrous pyridine (15 mL) at 0°C, under an atmosphere of N_2. The resulting yellow mixture was heated to reflux for 1 hour, allowed to cool down, poured into H_2O/ice (50 mL) and acidified with 5M

HCl. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (30 mL), 10% aq. Na_2CO_3 (30 mL), then brine (30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give an orange foam (250 mg). The crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 95:5) to give **121** as a pale yellow oil that crystallised on standing (140 mg, 47%) : mp 115-117°C; IR (KBr) 2930-2860 (aliph CH), 1770, 1725, 1655 (C=O), 1615, 1495 (aliph C=C and arom C=C), 1195 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.93 (3H, s, C-18-H_3), 1.42-2.84 (11H, m), 2.24 (3H, s, $COCH_3$), 2.29 (3H, s, $COCH_3$), 2.89-2.96 (2H, m, C-6-H_2), 6.81 (1H, d, J = 2.6 Hz, C-4-H), 6.86 (1H, dd, J = 8.3 Hz, J = 2.6 Hz, C-2-H), 7.29 (1H, d, J = 8.3 Hz, C-1-H) and 8.15 (1H, dd, J = 2.9 Hz, J = 1.7 Hz, C-1′-H); MS *m/z* (FAB+) 383.0 [100, $(M+H)^+$], 341.0 [86, $(M+H-CH_2CO)^+$]; Acc MS *m/z* (FAB+) 383.1852, $C_{23}H_{27}O_5$ requires 383.1858.

3-Acetoxy-6-oxo-16-acetoxymethylene-estra-1,3,5(10)-triene-17-one (122)

A solution of CrO_3 (132 mg, 1.32 mmol) in 10% AcOH (750 µL) was added dropwise over 30 minutes to a stirred solution of **121** (120 mg, 314 µmol) in AcOH glacial at 10-15°C in an ice/water bath. The resulting dark brown solution was stirred for 2 days at room temperature. The solvent was then removed under reduced pressure and H_2O (50 mL) and ice added. The organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a brown product. The crude material was purified by flash chromatography ($CHCl_3$/EtOAc, 8:2) to give **122** as a light yellow solid (13 mg, 10%): mp 96-101°C; IR (KBr) 2940-2860 (aliph CH), 1770, 1725, 1680, 1650 (C=O), 1615, 1490 (aliph C=C and arom C=C), 1195 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.95 (3H, s, C-18-H_3), 1.24-2.82 (10H, m), 2.26 (3H, s, $COCH_3$), 2.32 (3H, s, $COCH_3$), 2.88 (1H, dd, J_{AB} = 16.8 Hz, J = 3.5 Hz, C-7-H), 7.29 (1H, dd, J = 8.4 Hz, J = 2.6 Hz, C-2-H), 7.46 (1H, d, J = 8.4 Hz, C-1-H), 7.77 (1H, d, J = 2.6 Hz, C-4-H) and 8.17 (1H, dd, J = 2.9 Hz, J = 1.7 Hz, C-1′-H); MS *m/z* (FAB+) 663.5 [48], 397.2 [38, $(M+H)^+$], 355.2 [30, $(M+H-CH_2C=O)^+$], 73.0 [100]; Acc MS *m/z* (FAB+) 397.1670, $C_{23}H_{25}O_6$ requires 397.1651.

6-Oxo-16-ethoxymethylene-estrone (123)

A solution of KOH (27 mg, 482 μmol) in H_2O (0.8 mL) was added dropwise to a stirred solution of **122** (80 mg, 201 μmol) in EtOH at 0°C. The resulting bright yellow mixture was stirred at 0°C for 30 minutes, after which it was acidified with 5M HCl and the solvent removed under reduced pressure. H_2O was added (50 mL) and the organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a pale yellow product (52 mg). The crude material was purified by flash chromatography (DCM/EtOAc, 75:25) to give **123** as a white solid (12 mg, 18%): mp 314-316°C (dec.); IR (KBr) 3250 (OH), 2940-2860 (aliph CH), 1695, 1680 (C=O), 1620-1450 (aliph C=C and arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.94 (3H, s, C-18-H_3), 1.10-2.72 (10H, m), 1.35 (3H, t, J = 7.0 Hz, C-2″-H_3), 2.85 (1H, dd, J_{AB} = 16.7 Hz, J = 3.1 Hz, C-7-H), 4.05-4.13 (2H, m, C-1″-H_2), 5.10 (1H, s, C-3-OH), 7.09 (1H, dd, J = 8.5 Hz, J = 2.7 Hz, C-2-H), 7.31-7.34 (2H, m, C-1-H and C-1′-H) and 7.57 (1H, d, J = 8.5 Hz, C-4-H); MS *m/z* (FAB+) 341.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 341.1747, $C_{21}H_{25}O_4$ requires 341.1753. HPLC (MeOH/H_2O, 90:10, λ_{max} = 253.6 nm) Rt = 2.36 min, 100%.

6-Oxo-16-hydroxymethylene-estrone (124)

A solution of K_2CO_3 (63 mg, 456 μmol) in H_2O (1 mL) was added dropwise to a stirred solution of **122** (30 mg, 76 μmol) in MeOH (10 mL). The resulting pale yellow solution was stirred for 45 minutes, after which it was acidified with 5M HCl and the solvent removed under reduced pressure. H_2O was added (30 mL) and the organics were extracted with EtOAc (2×50 mL), washed with H_2O (2×30 mL), then brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The pale yellow crude product was recrystallised from EtOAc/hexane to give **124** as a white powder (13 mg, 54%): mp 224-227°C; IR (KBr) 3475, 3415 (OH), 2935-2860 (aliph CH), 1705, 1665 (C=O), 1605, 1495 (aliph C=C and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.80 (3H, s, C-18-H_3), 1.41-2.65 (11H, m), 7.01 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H), 7.28 (1H, d, J = 2.7 Hz, C-4-H), 7.31(1H, d, J = 8.4 Hz, C-1-H), 7.41 (1H, s, =C*H*OH), 9.64 (1H, s, exchanged with D_2O, C-3-OH) and 10.77 (1H, br s, exchanged with D_2O, =CHO*H*); MS *m/z* (FAB+) 313.2 [6, $(M+H)^+$], 149.1

[100]; MS *m/z* (FAB-) 311.2 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+) 313.1450, $C_{19}H_{21}O_4$ requires 313.1440. HPLC (MeOH/H_2O, 90:10, λ_{max} = 221.6 nm) Rt = 2.16 min, 100%.

3-Hydroxy-6,17-bis-oximino-estra-1,3,5(10)-triene (125)

NaOAc (293 mg, 3.58 mmol) followed by hydroxylamine hydrochloride (274 mg, 3.94 mmol) were added to a solution of **120** (100 mg, 352 μmol) in a mixture of MeOH/H_2O (5:1, 18 mL). The resulting solution was stirred at room temperature overnight, after which the solvent was removed under reduced pressure and H_2O added (50 mL). The organics were extracted with EtOAc (70 mL), washed with H_2O (2×20 mL), then brine (2×20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a light brown crude product (120 mg). This was purified by flash chromatography ($CHCl_3$/EtOAc, 7:3 to 1:1, gradient then $CHCl_3$/EtOAc/acetone, 2:2:1 to 1:1:2, gradient) to give **125** as an off-white powder (46 mg, 41%): mp 341-344°C; IR (KBr) 3410, 3265-3050 (br, NOH, OH), 2930-2850 (aliph CH), 1705 (C=N), 1580-1495 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.83 (3H, s, C-18-H_3), 1.14-2.42 (12H, m), 3.06 (1H, app dd, *J* = 17.8 Hz, *J* = 3.7 Hz, C-7-H), 6.73 (1H, dd, *J* = 8.3 Hz, *J* = 2.7 Hz, C-2-H), 7.14 (1H, d, *J* = 8.3 Hz, C-1-H), 7.29 (1H, d, *J* = 2.7 Hz, C-4-H), 9.28 (1H, s, exchanged with D_2O, OH), 10.15 (1H, s, exchanged with D_2O, NOH) and 11.08 (1H, s, exchanged with D_2O, NOH); MS *m/z* (FAB+) 315.1 [82, $(M+H)^+$], 73.0 [100]; Acc MS *m/z* (FAB+) 315.1715, $C_{18}H_{23}N_2O_3$ requires 315.1709. HPLC (MeOH/H_2O, 90:10; λ_{max} = 255.7 nm) Rt = 1.77 min, 98%.

3-Hydroxy-6,17-bis-(*O*-methyl-oximino)-estra-1,3,5(10)-triene (126)

NaOAc (146 mg, 1.79 mmol) followed by *O*-methyl-hydroxylamine hydrochloride (164 mg, 1.97 mmol) were added to a solution of **120** (50 mg, 176 μmol) in a mixture of MeOH/H_2O (5:1, 9 mL). The resulting solution was stirred at room temperature overnight, after which the solvent was removed under reduced pressure and H_2O added (30 mL). The organics were extracted with EtOAc (30 mL), washed with H_2O (2×10 mL) then brine (2×10 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The white crystalline crude product was recrystallised from EtOAc/hexane to give **126** as white crystals (55 mg, 92%): mp

206-208°C; IR (KBr) 3135 (br, OH), 2995 (arom CH), 2935-2890 (aliph CH), 1670, 1630 (CN, weak), 1570-1490 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.24-2.58 (12H, m), 3.12 (1H, dd, J_{AB} = 18.1 Hz, J = 4.5 Hz, C-7-H), 3.84 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 4.85 (1H, br s, exchanged with D_2O, OH), 6.84 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.20 (1H, d, J = 8.6 Hz, C-1-H) and 7.29 (1H, d, J = 2.7 Hz, C-4-H); MS *m/z* (FAB+) 343.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 343.2033, $C_{20}H_{27}N_2O_3$ requires 343.2022. Found: C, 70.20; H, 7.57; N, 8.14. $C_{20}H_{26}N_2O_3$ requires: C, 70.15; H, 7.65; N, 8.18%.

3-Hydroxy-17-oximino-estra-1,3,5(10)-triene (127)

NaOAc (1.5 g, 18.80 mmol) followed by hydroxylamine hydrochloride (1.4 g, 20.72 mmol) were added to a suspension of E1 (500 mg, 1.85 mmol) in a mixture of MeOH/H_2O (5:1, 90 mL). The resulting suspension was stirred at room temperature overnight, after which the solvent was removed under reduced pressure and H_2O added (100 mL). The organics were extracted with EtOAc (150 mL), washed with H_2O (50 mL), then brine (50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a white crystalline crude product (627 mg). This compound was recrystallised from MeOH to give **127** as white crystals (426 mg, 81%): mp 251-253°C (dec.) [lit.[155] (MeOH) 248-250°C]; IR (KBr) 3415 (NOH), 3270 (OH), 2930 (aliph CH), 1620 (C=N), 1585-1460 (arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.85 (3H, s, C-18-H_3), 1.32-2.41 (13H, m), 2.65-2.80 (2H, m, C-6-H_2), 6.44 (1H, d, J = 2.6 Hz, C-4-H), 6.50 (1H, dd, J = 8.5 Hz, J = 2.6 Hz, C-2-H), 7.05 (1H, d, J = 8.5 Hz, C-1-H), 9.01 (1H, s, exchanged with D_2O, OH) and 10.10 (1H, s, exchanged with D_2O, NOH); MS *m/z* (FAB+) 286.1 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 286.1809, $C_{18}H_{24}NO_2$ requires 286.1807. HPLC (MeOH/H_2O, 70:30; λ_{max} = 280.5 nm) Rt = 2.34 min, 98%.

3-Hydroxy-16,17-bis-oximino-estra-1,3,5(10)-triene (128)

NaOAc (556 mg, 6.79 mmol) followed by hydroxylamine hydrochloride (520 mg, 7.48 mmol) were added to a solution of **1** (200 mg, 668 μmol) in a mixture of MeOH/H_2O (5:1, 36 mL). The resulting pale yellow solution was stirred at room temperature overnight, after which the solvent was removed under reduced pressure and brine added (100 mL). The organics were extracted with EtOAc (100 mL),

washed with brine (2×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was recrystallised from acetone to give **128** as off-white crystals (109 mg, 52%): mp 245-247°C; IR (KBr) 3420-3200 (br, NOH, OH), 3020 (arom CH), 2935-2870 (aliph CH), 1705, 1620, 1585-1500 (C=N and arom C=C) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.97 (3H, s, C-18-H_3), 1.28-2.83 (13H, m), 6.44 (1H, d, J = 2.4 Hz, C-4-H), 6.51 (1H, dd, J = 8.5 Hz, J = 2.4 Hz, C-2-H), 7.04 (1H, d, J = 8.5 Hz, C-1-H), 9.02 (1H, s, exchanged with D_2O, OH), 10.81 (1H, s, exchanged with D_2O, NOH) and 11.19 (1H, s, exchanged with D_2O, NOH); δ_C (DMSO-d_6, 100.4 MHz) 14.5 (q, C-18), 26.5 (t), 27.1 (t), 27.3 (t), 29.5 (t), 35.5 (t), 37.4 (d), 43.4 (d), 46.2 (s, C-13), 49.0 (d), 113.2 (d), 115.4 (d), 126.4 (d), 130.5 (s), 137.4 (s), 155.5 (s), 155.7 (s) and 160.2 (s); MS *m/z* (FAB+) 315.2 [100, $(M+H)^+$]; MS *m/z* (FAB-) 466.2 [66, $(M-H-NBA)^-$], 313.2 [100, $(M-H)^-$], 276.1 [80]; Acc MS *m/z* (FAB+) 315.1711, $C_{18}H_{23}N_2O_3$ requires 315.1709. HPLC ($MeOH/H_2O$, 70:30, λ_{max} = 252.1 nm) Rt = 2.71 min, 99%. Found: C, 67.30; H, 7.41; N, 7.59. $C_{18}H_{22}N_2O_3.(CH_3)_2O$ requires: C, 67.72; H, 7.58; N, 7.52%.

5.4.8 Other sulfamates of E1 derivatives and precursors

3-Benzyloxy-16-ethoxymethylene-estra-1,3,5(10)-triene-17-one (129)

K_2CO_3 (595 mg, 4.31 mmol) was added portionwise to a stirred suspension of **72** (250 mg, 643 mmol) in acetone (20 mL) at room temperature, under an atmosphere of N_2. After 10 minutes of stirring, ethyl iodide (296 µL, 3.70 mmol) was added and the resulting mixture was stirred for 36 hours at room temperature. H_2O was then added (100 mL) and the organics were extracted with DCM (3×50 mL), washed with H_2O (50 mL), then brine (2×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc/hexane, 1:3) to give **129** as a white solid (215 mg, 80%). This compound was recrystallised from EtOAc to give white crystals (139 mg, 52%): mp 158-160°C; IR (KBr) 2935-2850 (aliph CH), 1710 (C=O), 1640-1500 (aliph and arom C=C), 1235 (C-O) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.92 (3H, s, C-18-H_3), 1.34 (3H, t, J = 7.2 Hz, C-2″-H_3), 1.38-2.71 (11H, m), 2.84-2.93 (2H, m, C-6-H_2), 4.02-4.10 (2H, m, C-1″-H_2), 5.02 (2H, s, OCH_2Ar), 6.71 (1H, d, J = 2.6 Hz, C-4-H), 6.77 (1H, dd, J = 8.6

Hz, J = 2.6 Hz, C-2-H), 7.18 (1H, d, J = 8.6 Hz, C-1-H) and 7.26-7.43 (6H, m, C-1′-H and C_6H_5); MS *m/z* (FAB+) 417.0 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 417.2425, $C_{28}H_{33}O_3$ requires 417.2430.

16β-Ethoxymethyl-estrone (64) and 16-ethoxymethyl-estradiol (130 and 131)

Following method 2, a suspension of **129** (640 mg, 1.54 mmol) and Pd-C (10%, 200 mg) in MeOH/THF (1:1, 60 mL) was hydrogenated for 48 hours. The crude mixture was purified by flash chromatography (EtOAc/hexane, 3:7) to give three products:

The less polar fraction gave **64** as a white crystalline solid (232 mg, 46%).

The following fraction gave **130** as a white crystalline solid (45 mg, 9%). An analytical sample was recrystallised from EtOH: mp 199-201°C; IR (KBr) 3415, 3240 (br, OH), 2972-2865 (aliph CH), 1610-1505 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.83 (3H, s, C-18-H_3), 1.12-2.51 (12H, m), 1.16 (3H, t, J = 7.0 Hz, C-2″-H_3), 2.79-2.82 (2H, m, C-6-H_2), 3.30 (1H, d, J = 4.7 Hz, exchanged with D_2O, C-17-OH), 3.45-3.61 (4H, m, C-1′-H_2 and C-1″-H_2), 3.85 (1H, dd, J = 9.9 Hz, J = 4.7 Hz, C-17-H), 4.79 (1H, s, exchanged with D_2O, C-3-OH), 6.54 (1H, d, J = 2.7 Hz, C-4-H), 6.60 (1H, dd, J = 8.4 Hz, J = 2.7 Hz, C-2-H) and 7.13 (1H, d, J = 8.4 Hz, C-1-H; MS *m/z* (FAB+) 331.3 [100, $(M+H)^+$], 272.1 [25, $(M+H-CH_2OEt)^+$], 145.1 [36], 85.0 [46]; Acc MS *m/z* (FAB+) 331.2291, $C_{21}H_{31}O_3$ requires 331.2273.

The more polar fraction gave **131** as a white crystalline solid (99 mg, 19%). An analytical sample was recrystallised from EtOH: mp 106-109°C; IR (KBr) 3315, 3215 (br, OH), 2977-2860 (aliph CH), 1620-1495 (arom C=C) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.84 (3H, s, C-18-H_3), 1.21 (3H, t, J = 7.0 Hz, C-2″-H_3), 1.25-2.31 (13H, m), 2.78-2.82 (2H, m, C-6-H_2), 3.37-3.59 (5H, m, C-1′-H_2, C-1″-H_2, C-17-H), 4.79 (1H, s, exchanged with D_2O, C-3-OH), 6.54 (1H, d, J = 2.7 Hz, C-4-H), 6.60 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H) and 7.13 (1H, d, J = 8.6 Hz, C-1-H); MS *m/z* (FAB+) 330.3 [100, $(M)^+$].

3-Sulfamoyloxy-16β-ethoxymethyl-estra-1,3,5(10)-triene-17-one (132)

Following method 3, reaction of **64** (100 mg, 304 μmol) with sulfamoyl chloride in DMA (1.5 mL) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 8:2) to give **132** as a colourless oil (124 mg, 100%): IR (KBr) 3380, 3285 (br, NH_2), 2970-2865 (aliph CH), 1730 (C=O), 1605-1495 (arom C=C), 1380, 1190 (SO_2) cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.89 (3H, s, C-18-H_3), 1.16 (3H, t, J = 7.0 Hz, C-2″-H_3), 1.40-2.43 (12H, m), 2.89-2.94 (2H, m, C-6-H_2), 3.43-3.50 (2H, m, C-1″-H_2), 3.61 (1H, dd, J_{AB} = 9.4 Hz, J = 3.7 Hz, C-1′-H_AH_B), 3.66 (1H, dd, J_{BA} = 9.4 Hz, J = 5.5 Hz, C-1′-H_AH_B), 4.87 (2H, s, exchanged with D_2O, SO_2NH_2), 7.04 (1H, d, J = 2.4 Hz, C-4-H), 7.07 (1H, dd, J = 8.4 Hz, J = 2.4 Hz, C-2-H) and 7.29 (1H, d, J = 8.4 Hz, C-1-H); δ_C ($CDCl_3$, 100.4 MHz) 12.9 (q, C-2″), 15.1 (q, C-18), 25.8 (2×t), 26.4 (t), 29.4 (t), 32.0 (t), 37.4 (d), 44.3 (d), 48.2 (s, C-13), 49.0 (d), 50.3 (d), 66.6 (t, C-1′ or C-1″), 69.3 (t, C-1′ or C-1″), 119.1 (d), 122.1 (d), 126.8 (d), 138.8 (s), 139.1 (s), 148.0 (s, C-3) and 220.8 (s, C=O); MS *m/z* (FAB+) 408.3 [39, $(M+H)^+$], 145.1 [63], 131.1 [61], 117.1 [57], 85.1 [100], 68.0 [50]; Acc MS *m/z* (FAB+) 408.1841, $C_{21}H_{30}NO_5S$ requires 408.1845. HPLC ($MeOH/H_2O$, 90:10, λ_{max} = 267.5 nm) Rt = 2.28 min, 99%. Found: C, 59.65; H, 6.84; N, 3.36. $C_{21}H_{29}NO_5S.(CHCl_3)_{1/6}$ requires: C, 59.48; H, 6.88; N, 3.28%.

3-Sulfamoyloxy-16-oximino-estra-1,3,5(10)-triene-17-one (133)

Following method 3, reaction of **1** (200 mg, 668 μmol) with sulfamoyl chloride in DMA (1.5 mL) was complete within 3 hours. The crude product was purified by flash chromatography ($CHCl_3$/acetone, 8:2) to give **133** as a white foam (93 mg, 37%). This compound was triturated with hexane to give a white powder (36 mg, 14%): mp 88-91°C; IR (KBr) 3370-3250 (NH_2, NOH), 2930-2850 (aliph CH), 1740 (C=O), 1635-1495 (C=N and arom C=C), 1380, 1185 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.90 (3H, s, C-18-H_3), 1.32-2.48 (11H, m), 2.78-2.94 (2H, m, C-6-H_2), 7.00 (1H, m, C-4-H), 7.03 (1H, dd, J = 8.6 Hz, J = 2.3 Hz, C-2-H), 7.36 (1H, d, J = 8.6 Hz, C-1-H), 7.92 (2H, s, exchanged with D_2O, SO_2NH_2) and 12.41 (1H, s, exchanged with D_2O, NOH); MS *m/z* (FAB+) 532.2 [32, $(M+H+NBA)^+$], 379.1 [100, $(M+H)^+$]; MS *m/z* (FAB-) 531.1 [40, $(M+NBA)^-$], 377.1 [100, $(M-H)^-$]; Acc MS *m/z* (FAB+)

379.1320, $C_{18}H_{23}N_2O_5S$ requires 379.1328. Found: C, 56.70; H, 6.02; N, 6.83. $C_{18}H_{22}N_2O_5S$ requires: C, 57.13; H, 5.86; N, 7.40%.

3,1′-Bis-sulfamoyloxy-estra-1,3,5(10)-triene-[17,16-c]-pyrazole (134) and 3-sulfamoyloxy-estra-1,3,5(10)-triene-[17,16-c]-pyrazole (135)

Following method 3, reaction of **98** (170 mg, 577 μmol) with sulfamoyl chloride in DMA (2 mL) was complete within 4 hours. The crude product was purified by flash chromatography ($CHCl_3$/EtOAc, 1:1) to give two products:

The less polar fraction gave **134** as a pale yellow solid (85 mg, 32%): mp 115-120°C; IR (KBr) 3440, 3410 (NH_2), 2930-2860 (aliph CH), 1635-1495 (C=N and arom C=C), 1385, 1185 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.99 (3H, s, C-18-H_3), 1.40-2.68 (11H, m), 2.85-2.90 (2H, m, C-6-H_2), 6.97 (1H, d, J = 2.3 Hz, C-4-H), 7.02 (1H, dd, J = 8.6 Hz, J = 2.3 Hz, C-2-H), 7.35 (1H, d, J = 8.6 Hz, C-1-H), 7.70 (1H, s, C-5′-H), 7.88 (2H, s, exchanged with D_2O, SO_2NH_2) and 8.30 (2H, s, exchanged with D_2O, SO_2NH_2); MS *m/z* (FAB+) 452.8 [100, M^+], 373.9 [80, $(M+H-SO_2NH_2)^+$], 171.0 [26], 116.0 [29]; Acc MS *m/z* (FAB+) 452.1164, $C_{19}H_{24}N_4O_5S_2$ requires 452.1188. HPLC (MeOH/H_2O, 80:20, λ_{max} = 220.4 nm) Rt = 1.95 min, 97%.

The more polar fraction gave **135** as a white solid (123 mg, 57%): mp 219-221°C IR (KBr) 3370, 3305 (NH_2), 2975-2860 (aliph CH), 1590, 1495 (C=N and arom C=C), 1370, 1195 (SO_2) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 1.17 (3H, s, C-18-H_3), 1.38-2.59 (11H, m), 2.85-2.90 (2H, m, C-6-H_2), 6.97 (1H, d, J = 2.7 Hz, C-4-H), 7.02 (1H, dd, J = 8.6 Hz, J = 2.7 Hz, C-2-H), 7.25 (1H, s, C-5′-H), 7.35 (1H, d, J = 8.6 Hz, C-1-H), 7.89 (2H, s, exchanged with D_2O, SO_2NH_2) and 11.99 (1H, br s, exchanged with D_2O, NH); MS *m/z* (FAB+) 747.3 [38, $(2M+H)^+$], 527.2 [80, $(M+H+NBA)^+$], 509.2 [30], 443.2 [28], 374.2 [100, $(M+H)^+$]; Acc MS *m/z* (FAB+) 374.1544, $C_{19}H_{24}N_3O_3S$ requires 374.1538. HPLC (MeOH/H_2O, 80:20, λ_{max} = 218.0 nm) Rt = 2.04 min, 100%.

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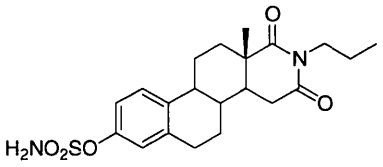
Appendix 1

X-ray crystallography data for **35**



Identification code	CCDC[a] 211567
Empirical formula	C21 H28 N2 O5 S
Formula weight	420.51
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 9.7920(3)Å $\alpha = 90°$
	b = 14.0950(3)Å $\beta = 90°$
	c = 14.6880(5)Å $\gamma = 90°$
Crystal size	0.20 × 0.17 × 0.08 mm

[a]Cambridge Crystallographic Data Centre

Notes:

NH_2 hydrogens located and freely refined.

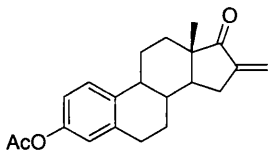
Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg:

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
N1-H1A	0.903	1.999	169.68	2.892	O2 [x+1/2, -y+3/2, -z+2]
N1-H2A	0.847	2.165	153.42	2.946	O5 [-x+5/2, -y+1, z+1/2]

For supplementary data, cf. enclosed CD (file name 'suppl. 35').

Appendix 2

X-ray crystallography data for **62**

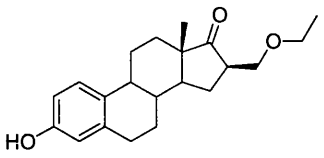


Identification code	h02phar2
Empirical formula	C21 H24 O3
Formula weight	324.40
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 9.29500(10)Å $\alpha = 90°$
	b = 10.10900(10)Å $\beta = 90°$
	c = 17.9620(2)Å $\gamma = 90°$
Crystal size	0.35 × 0.28 × 0.25 mm

For supplementary data, cf. enclosed CD (file name 'suppl. 62').

Appendix 3

X-ray crystallography data for **64**



Identification code	h03bp2
Empirical formula	$C_{21} H_{28} O_3$
Formula weight	328.43
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 7.1090(1)Å α = 90°
	b = 11.9830(2)Å β = 90°
	c = 21.4370(4)Å γ = 90°
Crystal size	0.40 x 0.13 x 0.04 mm

Notes:

H-bonded strands in lattice.

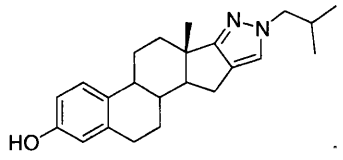
Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg:

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
O1-H1A	0.840	1.929	170.32	2.760	O2 [x, y+1, z]
N1-H2A	0.847	2.165	153.42	2.946	O5 [-x+5/2, -y+1, z+1/2]

For supplementary data, cf. enclosed CD (file name 'suppl. 64').

Appendix 4

X-ray crystallography data for **84**



Identification code	k02farm6
Empirical formula	$C_{24}H_{34}N_2O_2$
Formula weight	382.53
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 8.3600(1)Å α = 90°
	b = 13.2660(2)Å β = 90°
	c = 19.1680(4)Å γ = 90°
Crystal size	0.50 × 0.50 × 0.30 mm

Notes:

Hydrogen bonding in lattice.

Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg:

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
O1-H1	0.840	1.847	171.07	2.680	O2
O2-H2	0.840	1.911	172.59	2.746	N2' [x, y+1, z]

For supplementary data, cf. enclosed CD (file name 'suppl. 84').